Analysis of cell wall carbohydrate composition in *Eucalyptus* and *Arabidopsis*

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Abbreviations

°C	degrees Celsius
AIR	alcohol insoluble residue
anova	analysis of variance
Ara	arabinose
cont.	continued
CWM	cell wall material
DMSO	dimethyl sulphoxide
dp	decimal places
dx	delignified xylem
EDTA	ethylenediaminetetra acetic acid
EMS	ethylmethane sulphonate
Fuc	fucose
FW	fresh weight
g	grammes
G_2	cellobiose
Gal	galactose
GalA	galacturonic acid
Glc	glucose
GlcA	glucuronic acid
h	hours
ha	hectare
HPLC	high performance liquid chromatography
IP	isoprimeverose [xylosyl-α-(1→6)-D-glucose]
Km^R	kanamycin resistant
Km^S	kanamycin sensitive
Man	mannose
Mb	1×10^6 base pairs
mg	milligrammes

Contents

min	minutes
n.a.	not applicable
NaOH	sodium hydroxide
NASC	Nottingham *Arabidopsis* Stock Centre
NPTII	neomycin phosphotransferase
PAD	pulsed amperometric detector
PC	paper chromatography
Rha	rhamnose
Rib	ribose
sm	starting material
TFA	trifluoroacetic acid
TLC	thin layer chromatography
UV	ultra-violet
X_2	xylobiose [xylosyl-β-(1→4)-xylose]
Xyl	xylose

Abstract

The overall aim of the project was to use *Arabidopsis thaliana* as a model system to study the genes responsible for the biosynthesis of the major cell wall polymers. Information derived from *Arabidopsis* would be valuable in enabling the manipulation of the biosynthesis of the major cell wall polymers in industrially valuable tissues e.g. differentiating xylem cells of *Eucalyptus* trees.

Eucalyptus wood is used as a raw material for the pulp and paper industries. Lignin and non-cellulosic polysaccharides have to be removed from the wood during the production of cellulose pulp. Genetic modification of xylem could be of value in the development of wood that can be pulped with minimal use of polluting oxidants, or in the production of speciality papers. Depending upon the end-product, the presence of certain hemicellulosic polysaccharides can be beneficial (e.g. paper) or deleterious (e.g. cellophane, viscose rayons).

Eucalyptus xylem and pulp were analysed to ascertain the polysaccharides present in the wood pulp used in paper production and also the levels of these polysaccharides in the xylem of two *Eucalyptus* species (*E. grandis* and *E. globulus*). The methods used were Driselase digestion and trifluoroacetic acid (TFA) hydrolysis. Driselase digestion of cell walls produces disaccharides characteristic of some of the major cell wall polysaccharides, while hydrolysis with TFA yields monosaccharides. The analysis of the delignified xylem of the *Eucalyptus* spp. established that there was some natural variation between the species in the susceptibility of xylans to Driselase digestion, which probably reflects differences in the structure of these polysaccharides. Xylan was found to be a major contaminant of *Eucalyptus* wood pulp, making up approximately 25% of the mass of the pulp.

Various genotypes (T-DNA tagged mutants and Ds mutants) of *A. thaliana* were screened for altered cell wall polysaccharide composition. As an initial screen the products of the two assays (Driselase digestion and TFA hydrolysis) were separated by paper chromatography and thin layer chromatography. HPLC was then used to analyse lines that had shown consistent differences from the wild type in the initial screens. A small number of T-DNA tagged *A. thaliana* lines were identified that showed significant quantitative differences from the wild type in the composition of the screen products. The T-DNA tagged lines showed differences from the wild type in the amounts of cellulose, xyloglucan and xylan. Differences were also observed in the susceptibility of xylans to Driselase digestion, which probably reflect differences in the structure of these polysaccharides.

The presence of xylans and glucomannans has beneficial effects on paper properties; however, xylans are believed to inhibit the removal of residual lignin from pulp. It is possible that small changes in the level and/ or the composition of the xylan of the mutagenised *A. thaliana* lines may, once transferred to *Eucalyptus*, where xylan makes up a larger proportion of the total cell walls, affect the properties of the pulp produced from the modified trees.

1 Introduction

1.1 Primary cell walls

Primary cell walls are the basic structural framework of the growing plant; they control the rate of cell growth and form a physical barrier to pathogen attack (Darvill *et al.*, 1980, Bacic *et al.*, 1988). They are a major source of forage and food stuffs and as such are of economic importance.

The primary cell walls of angiosperms are composed primarily of carbohydrates and proteins (Albersheim, 1975; McNeil *et al.*, 1984; Carpita and Gibeaut, 1993). The carbohydrate component of the cell wall consists of cellulose microfibrils surrounded by a matrix of hemicelluloses and pectins.

1.1.1 Cellulose

The major carbohydrate of the primary wall is cellulose (Reiter, 1994). Cellulose is a β-(1→4)-linked glucan which forms a linear extended glucan chain with every other residue rotated 180° with respect to its neighbour, so cellobiose is the basic repeating unit (Delmer and Amor, 1995). The degree of polymerisation is between 2,000 and 6,000 (Delmer, 1987). Cellulose is present in the wall as crystalline microfibrils (5–12 nm in diameter; McCann *et al.*, 1992), which are aggregates of approximately 36 parallel chains (Albersheim, 1975; Delmer and Amor, 1995).

1.1.2 Hemicelluloses

1.1.2.1 Xyloglucan

Xyloglucan, a substituted β-(1→4)-linked glucan, is the most abundant hemicellulose in the primary wall of dicots (Hayashi, 1989; Reiter, 1994;). Xyloglucan molecules hydrogen-bond to cellulose microfibrils and may form cross links between different microfibrils (Fry, 1989). The xyloglucan from dicotyledonous primary cell walls has side chains attached to 60–70% of the glucose residues in the backbone (Bauer *et al.*, 1973; Hayashi

et al., 1980; Joseleau and Chambat, 1984; Hayashi, 1989). The major side chains are:

a) α-D-Xyl*p*-(1→6)- (Bauer *et al.*, 1973; Hayashi *et al.*, 1980; Joseleau and Chambat, 1984; Hayashi, 1989);

b) β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)- (York *et al.*, 1988; Kiefer *et al.*, 1989);

c) α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)- (Bauer *et al.*, 1973; Valent *et al.*, 1980; O'Neill and Selvendran, 1985)

These are often distributed in a repeating pattern of three consecutive xylosylated glucose residues followed by an unsubstituted glucose (Bauer *et al.*, 1973; Hayashi and Maclachlan, 1984). The galactose residues can be *O*-acetylated at O-6, O-3 or O-4 (York *et al.*, 1988; Kiefer *et al.*, 1989). There can be taxon-specific differences in the number and distribution of the side chains (Hayashi, 1989). A less common side chain is an α-(1→2) linked L-arabinofuranose residue attached to a glucose residue with an unsubstituted xylose residue attached (Hayashi, 1989; Kiefer *et al.*, 1990).

The structure of the xyloglucan molecule has been elucidated by the use of cellulases (fungal, e.g. *Trichoderma viride*, or plant; E.C. 3.2.1.4). These cellulases cleave the glucan backbone at the reducing end of unsubstituted glucose residues (Hayashi and Maclachlan, 1984). Owing to the structure of xyloglucan the major products of digestion are oligosaccharides of between 7 and 10 residues. A number of such oligosaccharides have been isolated and characterised (Bauer *et al.*, 1973; Hayashi *et al.*, 1980; Valent *et al.*, 1980; Hayashi and Maclachlan, 1984; O'Neill and Selvendran, 1985; Kiefer *et al.*, 1990).

1.1.2.2 Xylans

Xylans make up only about 5% of the primary wall of dicots (Reiter, 1994) but they are the predominant hemicelluloses in secondary cell walls (Section 1.2.3). The backbone of β-(1→4) linked xylose residues can be

substituted with (4-*O*-methyl) glucuronic acid residues linked to C-2 and arabinose residues linked to C-2 or C-3 of the xylose residues. The L-arabinose residues can be further substituted with β-D-galactose linked (1→5), β-D-xylose linked (1→2) or a galactose-xylose disaccharide (β-D-Gal*p*-(1→4)-β-D-Xyl*p*) linked (1→2) via the xylose residue to the arabinose residue (McNeil *et al.*, 1984). The major xylan in primary cell walls is a glucuronoarabinoxylan.

1.1.3 Pectins

Pectins are rhamnogalacturonans and homogalacturonans and can be partially extracted from the cell wall with hot water or aqueous solutions of chelating agents such as EDTA (Northcote, 1972; Reiter, 1994). Rhamnogalacturonans and homogalacturonans have backbones that contain a high proportion of α-D-galacturonic acid residues. The α-L-rhamnose residues, linked (1→2) in the backbone of the rhamnogalacturonans, produce kinks in the chain. A proportion of the galacturonic acid residues are methylesterified at the carboxyl group, or *O*-acetylated (Northcote, 1972). The negative charge of the acidic pectins allows Ca^{2+} crosslinks to form between different chains. Through the formation of 'egg box' junctions these crosslinks are thought to hold the acidic pectins together as a gel in the cell wall matrix (Jarvis, 1984; McNeil *et al.*, 1984; Carpita and Gibeaut, 1993). The formation of an egg box junction requires 25 uninterrupted galacturonic acid residues (Selvendran, 1985).

Rhamnogalacturonan I has a backbone composed of alternating 2-linked α-D-galacturonic acid and 4-linked α-L-rhamnose residues (McNeil *et al.*, 1984) and side chains that are made up of D-galactose, L-arabinose and small amounts of L-fucose and D-xylose. D-Glucuronic acid is also present as the terminal residue of a small proportion of the side chains, attached by β-(1→4) links to fucose and β-(1→6) links to galactose. A small amount of

xylose is attached by β-(1→3) links to the galacturonic acid residues of the main chain. Approximately half the rhamnose residues of rhamnogalacturonan I have an arabinan, galactan or arabinogalactan molecule bonded to them (Albersheim, 1975). These side chains are often highly branched and form blocks which are known as "hairy" regions, in contrast to the "smooth" regions of rhamnogalacturonan I which have no side chains.

Rhamnogalacturonan II is a highly branched complex polysaccharide. It contains a high proportion of rhamnose residues linked 3-, 3,4- and 2,3,4- as well as terminally (McNeil *et al.*, 1984). Rhamnogalacturonan II contains at least 12 monosaccharides, several of which are unusual (e.g. apiose and 3-deoxy-D-manno-2-octulosonic acid [KDO], Carpita and Gibeaut, 1993). The structure of rhamnogalacturonan II appears highly conserved between species (Stevenson *et al.*, 1988; Reiter, 1994).

Pectin deposition does not occur during secondary wall formation; however, pectins are present in mature lignified cell walls owing to their deposition in the primary cell wall (Imai and Terashima, 1992a). The exact amount present is difficult to quantify as once the wall is lignified the solubility of the uronic acids change and they become more tightly bound to hemicellulose (Ingold *et al.*, 1988) and lignin (Imai and Terashima, 1991). The main pectic substances in hardwoods are esterified homogalacturonan or rhamnogalacturonans (James *et al.*, 1985).

1.1.4 Structural proteins

Structural proteins in the walls of plant cells can be divided into five groups, reviewed by Showalter (1993). Two of the major classes of wall bound proteins are extensins and glycine-rich proteins (GRPs).

1.1.4.1 Extensins

Extensins are a family of hydroxyproline rich glycoproteins and, in dicotyledons, they usually contain the repeating motif Ser-Hyp_4. Short oligosaccharides are attached (via O-glycosylation) to hydroxyproline residues, often in a repeating block, to such an extent that carbohydrate can contribute over half the weight of the glycoprotein (Darvill *et al.*, 1980). Extensin is commonly associated with phloem and cambium cells, but it can be associated with other tissues as well (McNeil *et al.*, 1984). Extensin may form covalent crosslinks with other extensin molecules (McNeil *et al.*, 1984), and is thought to interact ionically with pectins. The deposition of extensin has been associated with wounding and infection responses.

1.1.4.2 Glycine-rich proteins

GRPs are characterised by an amino acid composition which can be up to 70% glycine arranged in short amino acid repeats. In dicotyledons GRPs are usually concentrated in vascular bundles, particularly xylem elements and have been associated with the initiation of lignification as well as wounding and infection responses.

1.1.5 Structure

The typical primary wall consists of about four layers of cellulose microfibrils coated with a monolayer of xyloglucan molecules which form cross links between the microfibrils (Albersheim, 1975; McCann *et al.*, 1992; Whitney *et al.*, 1995). This cellulose–xyloglucan network is the major load-bearing network in the primary cell wall. The cellulose microfibrils are often deposited transverse to the axis of elongation which forces turgor-driven cell expansion along the elongation axis (Reiter, 1994; Delmer and Amor, 1995). The xyloglucan molecules cross-linking the cellulose microfibrils can be up to 400 nm long and as the length of a cross link *in muro* is 20–40 nm long, one xyloglucan molecule is long enough to cross link several cellulose microfibrils (McCann *et al.*, 1992).

The pectins in the cell wall form a hydrated gel-like matrix cross linked through ionic Ca^{2+} bonds. This gel surrounds and may support the cellulose-xyloglucan network. The removal of pectin does not affect the integrity of the cellulose-xyloglucan network (McCann *et al.*, 1992) and Shedletzky *et al.* (1990; 1992) found that pectins can form a wall structure sufficient for cell growth and structural integrity in the virtual absence of a cellulose-xyloglucan network. There is evidence that pectins can be linked to non-carbohydrate components of the cell wall via ester bonds (Brown and Fry, 1993; Carpita *et al.*, 1993). The distribution of the different types of pectin is thought to vary within the cell wall. Rhamnogalacturonans are thought to be the major pectic component in the primary wall while homogalacturonans predominate in the middle lamella (McCann *et al.*, 1992, Selvendran, 1985).

1.2 Secondary cell walls

1.2.1 General

Once a cell has stopped growing it can differentiate into a specialised cell type e.g. vessel elements in xylem tissue and phloem fibres. This often involves the deposition of a cellulose-rich secondary wall on the innermost surface of the primary cell wall. Secondary cell walls are generally present in cells that are non living at maturity e.g. sclereids, fibres and vessel elements. During secondary growth there is an increased deposition of cellulose and hemicellulose on the inner surface of the existing primary wall, pectin deposition ceases and lignin synthesis may be initiated (Northcote, 1972; Cutter, 1978a; Ingold *et al.*, 1988). Once lignified, the secondary cell wall is mechanically stronger and more rigid than the primary wall (Whetten and Sederoff, 1995). The lignified wall also provides protection from some plant pathogens, which lack the necessary enzymes to degrade lignin (Cutter, 1978; Boudet *et al.*, 1995).

1.2.2 Cellulose

Cellulose accounts for approximately 40% of the dry weight of typical secondary walls (Delmer and Amor, 1995). Although in certain cell types the proportion can be much higher (approximately 94% in cotton seed hairs, Carpita and Gibeaut, 1993). The structure of secondary cell wall cellulose is typical of the cellulose found in primary walls although the cellulose microfibrils of the secondary wall can often be found further associated into macrofibrils or bundles (Timell, 1965; Delmer and Amor, 1995). The degree of polymerization of cellulose in the secondary wall is typically 8,000 to 10,000 (Delmer, 1987). The patterns of cellulose deposition during secondary growth can often form complex motifs, e.g helices or rings in the walls of the vessel elements of xylem tissue (Delmer and Amor, 1995).

1.2.3 Hemicelluloses

The predominant hemicellulose in secondary walls is a partially acetylated acidic xylan which can account for 20–30% of the dry weight of woody tissue (Bolwell and Northcote, 1981; James *et al.*, 1985). Glucomannans have also been found, although in limited amounts (Timell, 1965).

The acidic xylan is an *O*-acetyl-4-*O*-methylglucuronoxylan and it has been found in all hardwoods investigated (Timell, 1965; Preston, 1974). The backbone is (1→4)-linked β-D-xylopyranose residues, about 10% of which carry a 4-*O*-methyl-α-D-glucuronic acid residue glycosidically linked to the C2 position (Hazlewood and Gilbert, 1998). A proportion of the xylose residues (approximately 7 out of 10) are acetylated, mostly at C3 but to a certain extent also at C2 (Timell, 1965; Preston, 1974). Hardwood xylans are 150–200 xylose residues long (Preston, 1974).

The amount of xylan in the wall increases as cells differentiate and the onset of secondary thickening has been correlated with an increase in the activity of xylan synthetase (Dalessandro and Northcote, 1981; Ingold *et al.*,

1988; Imai and Terashima, 1992b). The amount of xylose in the cellulosic fraction also increases, indicating that some xylan is bound to the cellulosic fraction (Ingold *et al.*, 1988).

The glucomannan found in wood cell walls contains β-D-glucose and β-D-mannose linked (1→4) (Timell, 1965). The ratio of glucose to mannose is approximately 1:2.

1.2.4 Lignin

Lignin accounts for 15–36% of the dry weight of wood (Whetten and Sederoff, 1995). It reinforces the carbohydrate matrix of the cell wall, providing additional rigidity and compressive strength as well as rendering the walls hydrophobic and water-impermeable (Boudet *et al.*, 1995; Whetten and Sederoff, 1995).

In angiosperms lignins are polymers of two phenylpropanoid alcohols or monolignols (coniferyl alcohol and sinapyl alcohol) which are derived from phenylalanine. The dehydrogenative polymerisation of the monolignols is catalysed in the cell wall by wall bound peroxidases or oxidases. This results in the formation of 3-dimensional heteropolymers which surround the polysaccharides of the cell wall (Reiter, 1994; Whetten and Sederoff, 1995; Boudet *et al.*, 1995). The wide variety of linkages between the monolignols allows for a large amount of variability in the structure of lignin. Also, the monomer composition of lignin varies between and within species, among cell types within individual plants and even between different regions of the wall of a single cell (Lewis and Yamamoto, 1990; Whetten and Sederoff, 1995). The mechanisms that regulate monomer synthesis and lignin composition are still unclear. Reviews by Lewis and Yamamoto (1990) and Whetton and Sederoff (1995) cover the biosynthesis of lignin in detail, while the recent advances in the genetic manipulation of lignin and lignin biosynthesis are covered in a review by Boudet *et al.* (1995).

1.2.5 Structure

The secondary wall is composed of three layers (S_1, S_2 and S_3) which are deposited sequentially with S_3 innermost, and which differ in their polysaccharide and lignin composition (Timell, 1965; Cutter, 1978b; Boudet *et al.*, 1995). In birch, the middle lamella and the primary wall are rich in pectic material, S_3 and the inner S_2 have a high cellulose content while S_1 and the outer S_2 contain most of the xylan (Timell, 1965; Imai and Terashima, 1992b). After S_1 formation has begun and the deposition of pectic substances has finished, the first stage of lignification occurs at the cell corners and middle lamella. The second stage is a slow lignification associated with the deposition of cellulose microfibrils, mannan and xylan in S_2. The main lignification occurs in the third stage after the deposition of cellulose microfibrils in S_3 has started (Boudet *et al.*, 1995).

The differences in lignin composition in the three layers of the secondary wall may indicate that the kind of polysaccharide deposited before lignification is an important factor affecting the polymerisation of monolignols (Imai and Terashima, 1992b; Boudet *et al.*, 1995).

Lignin can be cross linked to the cell wall polysaccharides via ester linkages between uronic acids and hydroxyl groups of lignin monolignols on lignin surfaces, ether linkages between polysaccharides and lignin involving glucose or mannose residues, or glycosidic linkages between carbohydrates and terminal phenolic or side chain hydroxyls in lignin (Boudet *et al.*, 1995). Other linkages can also occur through *p*-coumaric acid and (in grasses) ferulic acid (Reiter, 1994; Boudet *et al.*, 1995). There is also evidence that lignins are associated with the hydroxyproline and glycine rich proteins of the cell wall (Boudet *et al.*, 1995).

Through the formation of links with lignin the polysaccharide component of the secondary wall may play an important role in the heterogeneous formation of the macromolecular structure of lignin and the lignin-carbohydrate complex (Imai and Terashima, 1990; Boudet *et al.*, 1995; Whetten and Sederoff, 1995).

Secondary cell walls, in the form of wood, are of significant economic importance in the pulp and paper industries because of the high percentage of cellulose present.

1.3 *Eucalyptus*

Eucalyptus wood provides an economically important source of timber, pulp and many secondary products such as oils and tannins (Wilkins and Horne, 1991). Eucalypts are hardwood evergreens and are among the fastest growing trees in the world. The moderate requirements needed to achieve fast growth have made certain eucalypts some of the world's most important plantation hardwoods (Wilkins and Horne, 1991). Two of the important eucalypts to the pulp and paper industry are *E. grandis* (Hill) Maiden and *E. globulus* Labill. *E. grandis* is one of the more frequently planted eucalypts because of its suitability for conversion into veneer, pulp, reconstituted products and sawn timber (Wilkins and Horne, 1991). The production of wood per year from *E. grandis* is 10–40 m^3/ ha, compared to 24–70 m^3/ ha for *E. globulus* (Pancel, 1993).

There are over 600 species of eucalypt, although some confusion exists because hybrids can occur in areas where several species grow together. Eucalypts can survive in a wide range of habitats from tropic to sub-temperate and from sea level to altitudes of up to 1,500 m. They can survive in areas with less than 25 cm of annual rainfall but are also found in regions with up to 3 m of annual rainfall.

1.3.1 Wood structure

The stem of a tree is made up of a column of xylem tissue surrounded by the cambium, which is itself bounded by phloem and cork. The cambium each year gives rise to additional xylem and phloem, causing an increase in the diameter of the stem. Xylem is the main water conducting tissue of

vascular plants. It extends throughout the body of the plant and is also involved in the transport of minerals, in food storage and in support. The secondary xylem of angiosperms is made up of vessels, tracheids, fibres, wood parenchyma cells and ray parenchyma cells.

1.3.1.1 Tracheids

Tracheids are elongated hollow cells with thickened, usually heavily lignified, walls, lacking protoplasts when mature. Tracheids have roles in conduction and mechanical support. Their lignified secondary walls are laid down in a variety of patterns, e.g. annular, sclariform, spiral, helical and reticulate (Esau, 1965). The end cell walls are not perforated but pits, usually bordered, are present. The pits of adjacent cells are opposite each other and water passes from cell to cell through these.

1.3.1.2 Vessel elements

Vessel elements are also elongated hollow cells with thickened, usually heavily lignified, walls, lacking protoplasts when mature. They are arranged end to end to form a vessel. The end walls of the elements are lost or perforated giving an open tubular structure. Vessels range from 20 to 400 μm or more in diameter and can be from a few cm to many metres long (Kramer and Kozlowski, 1960). Vessel elements can have secondary walls laid down in any of the patterns shown by tracheids.

1.3.1.3 Fibres

Usually the major part of woody tissue is made up of wood fibres. These are of negligible importance in conduction (Kramer and Kozlowski, 1960) but confer mechanical strength. Fibres resemble tracheids but have more extensive and heavier wall thickening and lignification (Esau, 1965). In fibres the pits are reduced in number and size, or are completely lost. Fibres lose their protoplast upon maturation.

1.3.1.4 Parenchyma

Parenchyma cells are present in xylem tissue as ray cells, but also occur interspersed amongst the tracheids and fibres. Wood rays are bands of parenchyma cells orientated horizontally, radiating outwards from the first annual ring. They are connected through the cambium to the phloem rays forming an important avenue for transport of carbohydrates and nutrients (Zimmerman and Brown, 1971). Wood ray and parenchyma cells have a storage function (Sporne, 1974). Parenchyma cells frequently possess a secondary cell wall and have simple pits. Upon maturation most of the parenchyma cells in the wood die leaving between 5 and 40% (Kramer and Kozlowski, 1960) alive in the sapwood, often for several years. The majority of these are part of the wood rays.

1.3.1.5 Heartwood

In fully differentiated xylem only the parenchyma cells are living and as a tree ages all the living cells of the central core die. Oils, gums, resins, tannins and other substances often accumulate in this heartwood causing it to become darker than the sapwood (Mauseth, 1988). The amount of heartwood formed depends upon the species and the site. Once heartwood starts to form it increases in diameter throughout the life of the tree. It is stronger, drier and more resistant to decay than sapwood. It provides mechanical support but does not participate in the physiological processes. Sapwood is much more important physiologically as its cells carry on metabolic processes, such as respiration and also store food and transport water and minerals.

1.4 Pulp production

The majority of pulp production is via one of two methods: the Kraft process and acidolysis (Glomb and Mulligan, 1989; Wallis and Wearne, 1992). According to Nissen *et al.* (1992) production of Kraft pulp is approximately 65–70 million tonnes per year. The Kraft pulping process reduces the lignin content of wood chips from around 30% to 3-4% and it is this residual lignin that gives the pulp its characteristic brown colour (Milagnes and Duran, 1992). The residual lignin can be removed by bleaching treatments. Both the Kraft process and acidolysis use large amounts of different chlorine compounds (chlorine, chlorite and hypochlorite) to bleach the pulp to a commercially acceptable colour (Glomb and Mulligan, 1989; Farrell and Skerker, 1992). Between bleaching stages the pulp is washed in an alkali solution to remove the by products of the bleaching step. These chlorinated organic compounds are known to have a toxic effect on the environment (Farrell and Skerker, 1992; Milagnes and Duran, 1992; Nissen *et al.*, 1992). This method is expensive, uses a large amount of water and generates a significant level of toxic waste products (Tien, 1987). Excess bleaching can degrade the pulp strength (Glomb and Mulligan, 1989), so a process by which the amount of chlorine required was reduced would be of importance to the industry.

There has been increasing concern for a number of years about the large amounts of toxic by-products of the pulping process (Tien, 1987; Nissen *et al.*, 1992). The pulp and paper industry is now beginning to modify its pulping, bleaching and effluent treatment technologies to reduce chlorine consumption. The aim is to eventually remove chlorine completely from the bleaching process without changing the physical qualities of the resulting paper (Nissen *et al.*, 1992; Wong and Saddler, 1992).

The monomer composition of lignin is a major factor in determining the difficulty of lignin degradation (Boudet *et al.*, 1995; Whetten and Sederoff, 1995). The guaiacyl-syringyl lignin typical of angiosperms is more easily removed by the Kraft pulping process than is the guaiacyl lignin

typical of conifers (Whetten and Sederoff, 1995). Cinnamyl alcohol dehydrogenase, a key enzyme in lignin biosynthesis, has been characterised in *Eucalyptus* (Goffner *et al.*, 1992; Grimapettenati *et al.*, 1993; Feuillet *et al.*, 1995; Samaj *et al.*, 1998) and in poplar and alfalfa (Vandoorsselaere *et al.*, 1995). Down-regulation of this enzyme results in an altered monomer composition of lignin (Halpin *et al.*, 1994).

Non-cellulosic polysaccharides such as pectins and hemicelluloses are intimately associated with cellulose and lignin and their presence in pulp can affect its physical and chemical properties (Imai and Terashima, 1990; 1991). In paper the presence of hemicelluloses have been shown to increase the tensile and bursting strength and also the folding endurance of the pulp (Wise and Lauer, 1962; Gamerith and Strutzenberger, 1992). For a specific type of paper there is often an optimal hemicellulose concentration (Wise and Lauer, 1962). Mannans appear to have a greater impact than xylans on certain strength properties and there is evidence that, up to a point, increasing the mannan content of a pulp will increase its tensile strength (Wise and Lauer, 1962). However, there are disadvantages to the presence of hemicellulose in pulps even when they are to be used for paper making. It appears that the hemicelluloses are, at least partly, responsible for a loss in brightness of certain bleached pulps on storage or ageing (Wise and Lauer, 1962) due to the presence of residual lignin. For the production of certain specialised products the presence of hemicelluloses are not desired (e.g. absorbent filter papers; Wise and Lauer, 1962) and they are considered to be an impurity in the pulps used to produce acetate products (such as viscose rayons and cellulose esters and ethers; Gardner and Chang, 1974). The hemicelluloses are not soluble in the solvents used and lead to defects in the final products as well as possibly damaging machinery. Certain xylan impurities can produce colour and haze defects and thermal instability in the acetate products while contaminating mannans can also produce haze defects (Wise and Lauer, 1962; Gardner and Chang, 1974; Wong and Saddler, 1992; 1993).

The lignin-xylan bond is one of the important linkages between lignin and carbohydrates and xylose has been found to be a major contaminant of Klason lignin (Imai and Terashima, 1992). The alkaline Kraft cooking of wood solubilises a portion of the xylan (or xylan-lignin complexes) in the cell wall which at a later stage of pulping precipitates on cellulose microfibrils and becomes insoluble (Biely *et al.*, 1992; Joseleau *et al.*, 1992). The xylan isolated from Kraft pulp presumably contains this reprecipitated xylan as well as xylan which has not been solubilised during cooking. Both types of xylan appear to form physical barriers to the extraction of residual lignin (Tenkanen *et al.*, 1992).

It has been suggested that the acetyl groups in xylan stabilise the xylan structure and prevent molecular orientation and development of lateral order (Nieduszynski and Marchessault, 1972; Tenkanen and Poutanen, 1992). The hemicelluloses remaining in the pulp (after cooking, bleaching and alkalisation) are completely deacylated and likely to hydrogen bond to cellulose microfibrils (Gamerith and Strutzenberger, 1992; Joseleau *et al.*, 1992). The strength of the hydrogen bonding between xylan and cellulose microfibrils is believed to be inversely proportional to the degree of substitution of the xylan molecules (Varner and Lin, 1989).

In considering future trends in bleaching xylanases are of interest in extending the delignification of pulp. This alternative approach is based on reports that residual lignin in unbleached Kraft pulp is linked to hemicellulose and that cleavage of this link will allow lignin to be released. (Davis *et al.*, 1992; Farrell and Skerker, 1992; Milagnes and Duran, 1992; Pekarovicová *et al.*, 1992). During trials of xylanase as a pre-chlorination treatment of Kraft pulps, it was found that the amount of chlorite needed to achieve the required brightness (a measure of how white the pulp is) was reduced by as much as 15% (Wong and Saddler, 1993; Davis *et al.*, 1992). It appears that treatment with xylanase, while solubilising the xylan present in the pulp, also solubilises any monolignols bound to that xylan. However, a portion of the xylan in pulp is inaccessible to xylanase treatment and this has

limited the potential of this application (Wong and Saddler, 1992).

The susceptibility of aspen wood and wheat straw xylans to enzymic degradation can be increased 5–7 times, and that of birch wood oligomers 2–3 times, by chemical deacetylation. However the removal of the acetyl substituents was shown to lead to the formation of xylan aggregates which were not as readily hydrolysed as the soluble acetylated substrate. Enzymic deacetylation (by acetyl xylan esterases) could not liberate the maximum theoretical amount of acetic acid. 4-O-methylglucuronic acid side groups adjacent to acetyl substituents have been reported to hinder the acetyl xylan esterase from liberating the remaining acetyl substituents (Tenkanen and Poutanen, 1992a).

A potential alternative to the use of the harsh chemical treatments or the use of enzymes in pulp production is to identify mutants which are altered such that the lignin and/ or hemicellulosic components of wood are present at decreased levels or do not require such extreme chemical conditions to extract them from the cellulose pulp.

1.5 Mutagenesis

Mutagenesis is the process of inducing any change in the genetic material of an organism, which is subsequently transmitted to daughter cells where it gives rise to a mutant cell or individual (Feldmann *et al.*, 1994). The use of chemical agents (e.g. ethylmethane sulphonate, nitrosoguanidine and nitrosourea) and ionizing radiation (e.g. X-rays) gives rise to point mutations, i.e. a change in one base pair of the DNA strand. The use of these mutagens has resulted in thousands of mutants in *Arabidopsis* (for lists of characterised mutants see Meyerowitz and Ma, 1994). Most of the mutations available in *A. thaliana* have been induced by chemical mutagens or some form of ionizing radiation (Feldmann *et al.*, 1994).

An alternative form of mutagenesis is insertional mutagenesis. Insertional mutagenesis is based on the inactivation of a gene via insertion of

a known DNA fragment. Insertional mutagens provide a relatively easy means of isolating the affected gene (Koncz *et al.*, 1990; Yanofsky *et al.*, 1990; Deng *et al.*, 1992; Kieber *et al.*, 1993; Tsay *et al.*, 1993). In plants either of two elements is generally used for mutagenesis: T-DNA and transposons.

1.5.1 T-DNA insertional mutagenesis

The T-DNA, or transfer DNA, of *Agrobacterium tumefaciens* is a defined segment of the tumour-inducing plasmid which, upon infection [by incubation of a bacterial culture with leaf disks (Koncz *et al.*, 1989) or seeds (Feldmann and Marks, 1987)] is transferred to a susceptible plant cell where it integrates into the nuclear genome (Forsthoefel *et al.*, 1992, Feldmann *et al.*, 1994). Once integrated the T-DNA genes are expressed and, depending upon where the integration occurs, a mutation may result such that the mutation and the marker (e.g. a gene for antibiotic resistance) in the T-DNA will co-segregate (Feldmann and Marks, 1987; Forsthoefel *et al.*, 1992; Koncz *et al.*, 1992).

1.5.2 Transposon Mutagenesis

The *Ac/Ds* transposon system from maize has been the most widely used for transposon mutagenesis (Bancroft and Dean, 1993; Long *et al.*, 1993; Altmann *et al.*, 1995). The *Ac* element is an autonomous element (i.e. it does not require the presence of any other element to transpose from one part of the genome to another) that codes for a transposase protein. The non-autonomous *Ds* element carries the sequences necessary for transposition in *trans*, i.e. it can only transpose in the presence of another element which provides the functional transposase protein. Mutagenesis strategies have used either the single autonomous *Ac* element or a two-element system with Ds *trans*-activated by an 'inactivated' *Ac* element that is not capable of transposition. Re-integration of the *Ds* elements into the genome can be selected for by including a dominant selectable marker within the *Ds*

element such as antibiotic resistance (Bancroft *et al.*, 1992; 1993; Bancroft and Dean, 1993).

1.6 *Arabidopsis thaliana*

1.6.1 A model system

Arabidopsis thaliana (a member of the Brassicaceae family) has a number of characteristics which make it a useful model system for genetic and biochemical studies. It has a life cycle of only 6 to 8 weeks which allows a number of generations to be grown in a relatively short period. The small size of the plant means that a large number can be grown under controlled conditions in a small area. Outcrossing occurs at a very low frequency (1-2%) and a single plant, under optimal conditions, can produce over 20,000 seeds within a few weeks (Koornneef, 1994). These characteristics mean that handling the plant for classical genetic purposes is not very labour intensive.

A. thaliana has an unusually small nuclear genome for a flowering plant (approximately 100 Mb) and has remarkably little dispersed repetitive DNA (10-14%; Meyerowitz, 1994). These properties facilitate molecular genetics techniques. *A. thaliana* also has a low chromosome number (2n = 10) which allows for a more efficient linkage analysis than in plants with more chromosomes (Price *et al.*, 1994). Despite the unusual size and structure of the *Arabidopsis* nuclear genome, the structure of individual genes, the structure of chromosomes, the genetic properties, and the overall complement of genes in the genome are typical of those of other flowering plants (Meyerowitz, 1994).

1.6.2 *A. thaliana* cell wall mutants

A large number of mutations in *A. thaliana* have been identified that affect various developmental and physiological processes such as flower development and hormone action (for lists of characterised mutants see

Meyerowitz and Ma, 1994). However, only a small number of *A. thaliana* mutants with defective cell wall metabolism have been isolated. The cell wall mutants have been identified either by analysis of the monomer composition of the wall, by screening for a specific enzyme activity, or by screening for a specific physical phenotype.

1.6.2.1 Mutants with altered monomer composition

The developmental pattern and biochemical nature of wild-type *Arabidopsis* lignin has been found to be typical of higher plants (Dharmawardhana *et al.*, 1992). Chapple *et al.* (1992) have identified an *Arabidopsis* mutant with altered phenylpropanoid metabolism (*sin1;* now renamed *fah1* to avoid confusion with another mutant previously referred to as *sin1*, Chapple *et al.*, 1994), from an EMS-mutagenised population, such that it has insignificant levels of syringyl-derived units in its lignin.

Zablackis *et al.* (1995) examined the leaf cell wall polysaccharides of *A. thaliana* as a preliminary step towards the identification of lines with altered cell wall polysaccharides. The composition and structure of the wall polysaccharides appeared to be typical of those found in other plants, apart from a high proportion of phosphate buffer-soluble pectic polysaccharides. The leaf cell walls were found to contain six types of polysaccharide: homogalacturonan, rhamnogalacturonan-I, rhamnogalacturonan-II, glucuronoarabinoxylan, xyloglucan and cellulose (Zablackis *et al.*, 1995). Reiter *et al.* (1993, 1997; Zablackis *et al.*, 1996) have identified 11 lines with altered monosaccharide composition (designated *mur*) from an EMS-mutagenised population of *A. thaliana* ecotype Columbia. These lines have a range of phenotypes, with alterations being observed in the levels of fucose, arabinose, rhamnose, xylose and mannose. The best characterised locus is *mur1* (Reiter *et al.*, 1993). The leaves and bolting stem of the *mur1* plant were found to have less than 2% of the wild type level of fucose. The activity of an isoform of GDP-D-mannose-4,6-dehydratase is believed to be affected, resulting in a deficiency in production of GDP-L-fucose. This in turn results

in the partial replacement of the terminal L-fucose in the side chains of xyloglucan with L-galactose (Zablackis *et al.*, 1996; Bonin *et al.*, 1997). The plant has a dwarfed growth habit and the bolting stems require a much reduced force to break them (Reiter *et al.*, 1993). The terminal L-fucose of the side chains of xyloglucan has been implicated in determining the cellulose-binding properties of xyloglucan (Hayashi, 1989).

1.6.2.2 Mutants with deficient enzyme activity

An arabinose-sensitive mutant was identified by Dolezal and Cobbett (1991) from an EMS-mutagenised population of *A. thaliana* ecotype Columbia. In the absence of arabinose the phenotype of the mutant line is indistinguishable from that of wild-type. However, when supplied with a low concentration of arabinose exogenously, the plants turn brown and necrotic within 2 days of germination. The mutation is believed to affect arabinose kinase, which is part of the arabinose salvage pathway. The loss of this enzyme allows arabinose to build up to toxic levels in the plant (Cobbett *et al.*, 1992).

1.6.2.3 Mutants with altered physical phenotypes

A mutant with a reduced ability to initiate and deposit secondary cell wall cellulose in leaf and stem trichomes (*tbr*) has been isolated by Potikha and Delmer (1995) from an EMS-mutagenised population of *A. thaliana* ecotype Columbia. Turner and Somerville (1997) have identified three lines which show a collapsed xylem phenotype (*irx*). The bolting stems of *irx* plants are weaker and much less stiff than those of wild-type plants and all three lines have significantly reduced levels of cellulose in the secondary cell walls (Turner and Somerville, 1997). These lines have a similar phenotype to the brittle culm mutants of barley, which have reduced cellulose in the cell walls and show a twofold reduction in breaking strength (Kokubo *et al.*, 1989, 1991).

It is possible that mutations affecting polysaccharide synthases, such that a plant would be completely deficient in one of the major cell wall polysaccharides (e.g. cellulose), would render a plant unable to grow normally owing to a reduction in the mechanical strength of the cell wall and an inability of the walls to extend during turgor-driven growth. These mutations would in effect be lethal.

Two genes (*Meri*-5, Medford *et al.*, 1991; and *TCH4*, Braam and Davis, 1990) were described based on their tissue-specific expression pattern and their inducibility by mechanical stimuli and later found to encode proteins with xyloglucan endotransglycosylase (XET) activity (Xu *et al.*, 1995). XETs are capable of cleaving the xyloglucan backbone and re-attaching one end to another xyloglucan molecule. XET has been implicated in the mechanism of cell wall loosening as part of turgor-driven wall expansion. Recent database searches have revealed the presence of at least 13 XET-related genes in *Arabidopsis* and XET activity has been demonstrated for four of these (Xu *et al.*, 1995, 1996).

1.7 Aim

The overall aim of the present project was to screen a mutagenised population of *A. thaliana* for lines that had quantitative or qualitative alterations in the cell wall polysaccharides that are present in paper or have an impact on the extraction and processing of wood in terms of the ease of extractability and/ or use of bleaching agents. The model system *A. thaliana* would be used to investigate the mutation and help to elucidate the biosynthesis and/ or the assembly of the cell walls. The information gathered would then be extrapolated to *Eucalyptus* where its impact on the processing of wood for the pulp and paper industries would be assessed.

2 Materials and Methods

2.1 General Methods

2.1.1 Purification of Driselase

Driselase (from the fungus *Irpex lacteus*, Sigma) is a mixture of endo- and exo-enzymes. The enzyme activities present include α-D-galactopyranosidase, β-D-galactopyranosidase, β-D-glucopyranosidase, α-D-mannopyranosidase, β-D-mannopyranosidase, α-L-arabinofuranosidase, β-D-xylopyranosidase, α-L-fucopyranosidase, cellulose-cellobiohydrolase, cellulase [β-(1→4)-D-glucanase], β-D-galactanase, α-L-arabinanase, pectinase [α-(1→4)-D-galacturonanase], β-D-mannanase, xylanase [β-(1→4)-D-xylanase], and an endo-(1→4)-β-glucanase that attacks all the β-glucose bonds of xyloglucan whether or not they are substituted by α-xylose residues.

This method was taken from Fry (1988). All procedures were carried out at 4°C. Driselase (5 g) was added to 50 ml of buffer A (50 mM acetic acid, adjusted to pH 5.0 with 1 M NaOH) and stirred for 15 min to dissolve the enzyme. The solution was centrifuged at 2,500 *g* for 10 min and the supernatant collected (if still cloudy the solution was recentrifuged and the pellet rejected). The supernatant was added to solid $(NH_4)_2SO_4$, 26 g per 50 ml of supernatant, and stirred constantly until the crystals were dissolved. The solution was left to stand for 15 min before being centrifuged (10 min at 2,500 *g*). The supernatant was rejected unless cloudy, in which case it was recentrifuged and the pellet kept. The pellet was resuspended in 50 ml of fresh 52% (w/v) $(NH_4)_2SO_4$ and recentrifuged for 10 min at 2,500 *g*. The pellet was redissolved in 5 ml water and desalted on a gel permeation column (Sephadex G-25) run in water. The protein was collected and freeze dried. The enzyme was stored below 0°C.

2.1.2 Preparation of isoprimeverose and xylobiose for marker solutions

To prepare marker solutions of isoprimeverose (xylosyl-α-(1→6)-glucose) and xylobiose (xylosyl-β-(1→4)-xylose) for paper chromatograms (PC) and thin layer chromatograms (TLC), a 1% solution of tamarind xyloglucan (for isoprimeverose, the generous gift of Mr. K. Yamatoya, Dainippon Pharmaceutical Co., Osaka, Japan) or birchwood xylan (for xylobiose, from Sigma) was incubated with 0.1% purified Driselase in pyridine/ acetic acid/ water (1:1:98, pH 4.7) at 37°C, on a shaker, for 48 h. Before incubation chlorobutanol (an antibacterial agent) was added to a concentration of 0.5%. PC and TLC markers were stored at 4°C.

For preparation of high performance liquid chromatography (HPLC) standards, the digestion products were loaded onto Whatman 3MM paper as a streak. Digestion products were separated by a solvent mixture of ethyl acetate/ pyridine/ water (8:2:1). Markers were stained with aniline hydrogen-phthalate (Section 2.2.7). Isoprimeverose (or xylobiose) was eluted from the paper with distilled water by centrifugation in a syringe inserted into a test tube (Eshdat and Mirelman, 1972). The eluate was collected in the test tube. The solution was freeze-dried, redissolved in water to give the desired concentration and filtered through a 0.2-μm Gelman Supor Acrodisc filter. HPLC standards were stored below 0°C.

2.1.3 Ethanol precipitation of polymers

Ethanol precipitates most biological polymers out of aqueous solution. Five volumes of absolute ethanol were added to one volume of sample, left at -20°C overnight then centrifuged and the pellet discarded.

This procedure was used to precipitate Driselase from aqueous solution before separation of the monosaccharide and disaccharide digestion products by HPLC.

2.1.4 Delignification

The xylem from *Eucalyptus* stems (approximately 1 year old) was delignified using this method (Whistler and BeMiller, 1963). *Eucalyptus* xylem (freeze-dried and ground, Section 2.2.3.2) in water (100 ml water to 1 g freeze-dried xylem) was placed in a water-bath at 70–75°C. Four aliquots of 0.28 ml glacial acetic acid and 0.8 g sodium chlorite were added at intervals of 15 min. The sodium chlorite was added slowly over several minutes following the addition of the acetic acid. The entire process was carried out in an open beaker in a fume hood to prevent chlorine dioxide, produced by the reaction, reaching an explosive mixture. Foaming was controlled when necessary by the addition of 1–2 drops of octan-1-ol. After 1.5 h in the water bath, the reaction mixture was cooled to 20–25°C and filtered. The residue was washed in water until the washes had a neutral pH, then washed in acetone and dried over phosphorus pentoxide.

2.1.5 Starch extraction

The following method (Selvendran *et al.*, 1985) was used to extract starch from the alcohol insoluble residue (AIR) of *Arabidopsis thaliana* lines. The AIR (Section 2.2.3.3) was stirred overnight with dimethyl sulphoxide (100 ml 90% DMSO to 10 g AIR) at 25°C. The residue was then washed with 50 ml 90% DMSO. This procedure was repeated until the residue was judged to be starch free. This was demonstrated by the addition of a drop of 0.33% iodine/ 0.67% potassium iodide to a small amount of DMSO-insoluble residue (washed in water). Any remaining starch stained blue-black. To remove the DMSO, the starch-free residue was dialysed against 70% ethanol. The residue was washed with 100% acetone and air dried.

2.2 Screening methods

2.2.1 Plant Species

2.2.1.1 *Eucalyptus* species

E. grandis plants and *E. globulus* seeds were supplied by Dr. M.M. Burrell of Advanced Technologies (Cambridge) Ltd.

2.2.1.2 *Arabidopsis thaliana* lines

The mutagenised lines of *A. thaliana* were supplied by the Nottingham *Arabidopsis* Stock Centre (NASC). The batches of lines obtained are listed below.

T-DNA tagged form-mutants from:

organ transformation (Koncz, 1992)

seed transformation (Feldmann and Marks, 1987; Feldmann *et al.*, 1989)

Ds-tagged lines (Bancroft & Dean, 1993)

See Table 2.1, Table 2.2 and Table 2.3 for lists of the lines obtained. All of the tagged lines obtained from the NASC were T4 generation. *A. thaliana* ecotype Wassilewskija (Ws, Feldmann parental line, NASC No. N1601) and *A. thaliana* ecotype Landsberg *erecta* (Bancroft and Dean parental line, NASC No. NW20) were also obtained from NASC. *A. thaliana* ecotype Columbia (Koncz parental line) was supplied by Dr. M.M. Burrell of Advanced Technologies (Cambridge) Ltd.

Feldmann lines

The Feldmann lines were produced by transformation of *A. thaliana* (L.) Heynh ecotype Wassilewskija (Ws) with *Agrobacterium tumefaciens* strain C58C1rif containing the Ti plasmid 3850:1003 (Feldmann and Marks, 1987). The transformation event resulted in the insertion of the T-DNA region of the Ti plasmid into the nuclear genome of the plant (Feldmann and Marks, 1987; Feldmann *et. al.*, 1989). The Ti plasmid 3850:1003 has the neomycin

phosphotransferase gene (NPT II) contained between the two Ti plasmid borders. NPT II confers resistance to kanamycin, which interferes with protein synthesis. The T2 generation was selected by kanamycin resistance, therefore the T4 population will be 75% kanamycin resistant plants and 25% kanamycin sensitive plants (Figure 2.1).

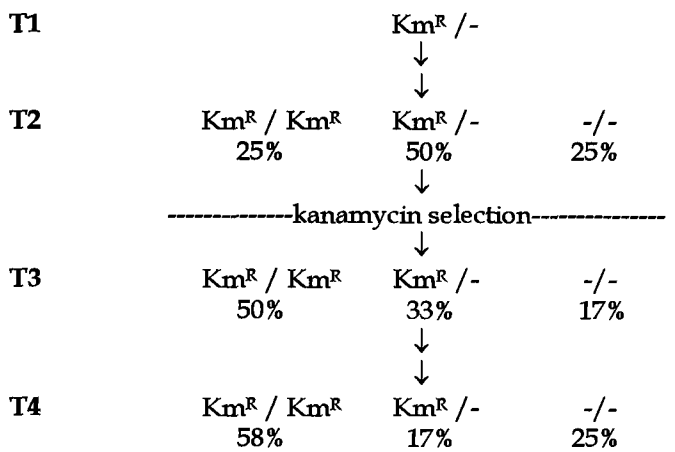

Figure 2.1 The proportion of Km^R alleles in a population of T-DNA tagged Feldmann lines assuming no outcrossing occurs. T1 to T4 = generation number, Km^R = kanamycin resistance, percentages are the proportion of each genotype in the population at each generation.

Table 2.1 T-DNA tagged lines (Koncz, 1992) obtained from the Nottingham *Arabidopsis* stock centre (NASC).

NASC No.	Screen No.	NASC No.	Screen No.	NASC No.	Screen No.	NASC No.	Screen No.	NASC No.	Screen No.
N4000	A1	N4037	A9	N4093	A17	N4222	A25	N4279	A33
N4011	A2	N4049	A10	N4111	A18	N4225	A26	N4284	A34
N4012	A3	N4050	A11	N4118	A19	N4228	A27	N4286	A35
N4019	A4	N4053	A12	N4122	A20	N4253	A28	N4291	A36
N4021	A5	N4067	A13	N4123	A21	N4262	A29	N4088	A38
N4025	A6	N4070	A14	N4144	A22	N4267	A30	N4153	A44
N4026	A7	N4078	A15	N4210	A23	N4268	A31	N4178	A45
N4027	A8	N4086	A16	N4219	A24	N4272	A32	N4200	A47

Table 2.2 T-DNA tagged lines (Feldmann and Marks, 1987; Feldmann *et al.*, 1989) obtained from the NASC.

NASC No.	Screen No.	NASC No.	Screen No.	NASC No.	Screen No.	NASC No.	Screen No.	NASC No.	Screen No.
N2844	B1	N2861	B17	N2877	B33	N2893	B49	N2910	B66
N2845	B2	N2862	B18	N2878	B34	N2895	B51	N2911	B67
N2846	B3	N2863	B19	N2879	B35	N2896	B52	N2912	B68
N2847	B4	N2864	B20	N2880	B36	N2897	B53	N2913	B69
N2848	B5	N2865	B21	N2881	B37	N2898	B54	N2914	B70
N2849	B6	N2866	B22	N2882	B38	N2899	B55	N2915	B71
N2850	B7	N2867	B23	N2883	B39	N2900	B56	N2916	B72
N2851	B8	N2868	B24	N2884	B40	N2901	B57	N2917	B73
N2852	B9	N2869	B25	N2885	B41	N2902	B58	N2919	B75
N2853	B10	N2870	B26	N2886	B42	N2903	B59	N2920	B76
N2854	B11	N2871	B27	N2887	B43	N2904	B60	N2921	B77
N2855	B12	N2872	B28	N2888	B44	N2905	B61	N2922	B78
N2857	B13	N2873	B29	N2889	B45	N2906	B62	N2923	B79
N2858	B14	N2874	B30	N2890	B46	N2907	B63	N2924	B80
N2859	B15	N2875	B31	N2891	B47	N2908	B64		
N2860	B16	N2876	B32	N2892	B48	N2909	B65		

Table 2.3 Ds-tagged lines (Bancroft & Dean, 1993) obtained from the NASC.

NASC No.	Screen No.	NASC No.	Screen No.	NASC No.	Screen No.	NASC No.	Screen No.	NASC No.	Screen No.
N4301	C1	N4351	C35	N4398	C69	N4453	C103	N4494	C137
N4302	C2	N4352	C36	N4400	C70	N4454	C104	N4595	C138
N4303	C3	N4353	C37	N4401	C71	N4455	C105	N4505	C139
N4304	C4	N4354	C38	N4402	C72	N4456	C106	N4507	C140
N4305	C5	N4355	C39	N4403	C73	N4457	C107	N4508	C141
N4306	C6	N4356	C40	N4404	C74	N4458	C108	N4509	C142
N4307	C7	N4357	C41	N4405	C75	N4459	C109	N4510	C143
N4308	C8	N4358	C42	N4406	C76	N4460	C110	N4512	C144
N4309	C9	N4359	C43	N4407	C77	N4461	C111	N4513	C145
N4310	C10	N4360	C44	N4408	C78	N4463	C112	N4517	C146
N4311	C11	N4361	C45	N4409	C79	N4464	C113	N4521	C147
N4312	C12	N4362	C46	N4411	C80	N4465	C114	N4523	C148
N4313	C13	N4363	C47	N4412	C81	N4466	C115	N4524	C149
N4315	C14	N4367	C48	N4414	C82	N4467	C116	N4525	C150
N4316	C15	N4368	C49	N4415	C83	N4470	C117	N4526	C151
N4317	C16	N4370	C50	N4419	C84	N4471	C118	N4527	C152
N4320	C17	N4371	C51	N4420	C85	N4472	C119	N4528	C153
N4323	C18	N4374	C52	N4421	C86	N4474	C120	N4531	C154
N4325	C19	N4375	C53	N4424	C87	N4475	C121	N4534	C155
N4329	C20	N4377	C54	N4426	C88	N4476	C122	N4535	C156
N4330	C21	N4379	C55	N4428	C89	N4477	C123	N4536	C157
N4332	C22	N4381	C56	N4429	C90	N4478	C124	N4537	C158
N4333	C23	N4382	C57	N4430	C91	N4480	C125	N4538	C159
N4335	C24	N4384	C58	N4432	C92	N4481	C126	N4539	C160
N4336	C25	N4386	C59	N4433	C93	N4482	C127	N4322	C161
N4338	C26	N4387	C60	N4438	C94	N4483	C128	N4328	C162
N4340	C27	N4389	C61	N4439	C95	N4484	C129	N4337	C163
N4341	C28	N4390	C62	N4440	C96	N4485	C130	N4343	C164
N4342	C29	N4391	C63	N4442	C97	N4486	C131	N4373	C165
N4345	C30	N4392	C64	N4445	C98	N4487	C132	N4529	C166
N4346	C31	N4393	C65	N4446	C99	N4490	C133	N4533	C167
N4347	C32	N4394	C66	N4448	C100	N4491	C134		
N4348	C33	N4396	C67	N4450	C101	N4492	C135		
N4349	C34	N4397	C68	N4452	C102	N4493	C136		

2.2.2 Growth conditions

2.2.2.1 *Eucalyptus* species

E. globulus and *E. grandis* were grown in soil, in a greenhouse with a day length of 18 h (3,000 lumen/ m^2) and an average day and night temperature of 25°C.

2.2.2.2 *A. thaliana*

Surface sterilisation of seeds

Seeds were surface sterilised (Dr. O. Leyser, University of York, private communication) before being sown on agar plates. Seeds were soaked for 15 min in 0.01% Triton X-100 in 10% sodium hypochlorite, washed in 70% ethanol and then washed in 4 changes of sterile water.

Soil method

Seeds were scattered on the surface of autoclaved compost (Fisons F2) soaked in ATS nutrient solution. Pots were covered with a propagator lid to prevent moisture loss and increase humidity, and placed in a growth room (12 h day [4,000 lumen/m^2]/night, 22°C day/night). Seedlings germinated after 3–5 days and plants began to produce flowering stems after 3–4 weeks. Plants were watered with ATS nutrient solution as required.

Agar method

Sterile seeds were sown, under aseptic conditions, on media containing 0.8% agar and 1% sucrose in ATS solution. The plates were placed in a growth room set at a 12 h day and a temperature of 22°C day and night.

ATS nutrient solution

The ATS nutrient solution (Dr. O. Leyser, University of York, private communication) was used for both soil and agar grown plants. The composition of ATS is given in Table 2.4.

Table 2.4. Composition of ATS nutrient solution

Compound	[Stock]	ml stock/ 1 ATS
KNO_3	1 M	5
KH_2PO_4	1 M	2.5
$Ca(NO_3)_2$	1 M	2
Fe^{3+}.EDTA	20 mM	2.5
$MgSO_4$	1 M	2
micronutrients:		1
H_3BO_3	70 mM	
$MnCl_2$	14 mM	
$CuSO_4$	0.5 mM	
$ZnSO_4$	1 mM	
$NaMoO_4$	0.2 mM	
NaCl	10 mM	
$CoCl_2$	10 µM	

2.2.3 Cell wall preparations

2.2.3.1 *Eucalyptus* pulp

Card produced from *Eucalyptus* sp. pulp was supplied by Dr. M.M. Burrell of Advanced Technologies (Cambridge) Ltd. The card was cut into small pieces, which were stirred in water (1 g of pulp in 100 ml water) to form a suspension.

2.2.3.2 *Eucalyptus* xylem

The stems from *Eucalyptus* plants (*E. grandis* and *E. globulus*, approximately 1 year old) were freeze-dried and stripped of the cork and phloem layers. The tissue was frozen in liquid nitrogen and partially broken up in an Ato Mix (a variable speed blender with metal blades, manufactured by MSE), cooled in ice prior to use, before being ground in a pestle and mortar. The ground xylem was delignified as described in Section 2.1.4 and dried.

2.2.3.3 Alcohol insoluble residue of *A. thaliana*

The screening methods used were Driselase digestion and TFA hydrolysis. The substrate for the screens was the AIR of the flowering stem of *A. thaliana*. This method was adapted from Fry (1988). This version was less labour intensive and allowed a large number of lines to be processed at once. Extraction in ethanol was carried out to remove low molecular weight sugars, amino acids, organic acids and many inorganic salts. The AIR was expected to be composed of protoplasmic proteins, RNA and starch as well as the wall polymers. However, many of these contaminants could be ignored for the purposes of these screens.

Primary flowering stems were cut once the first siliques had begun to form. This allowed for maximum growth before the tissue was collected. The leaves and side shoots were removed from the flowering stems before preparation of the AIR. Fresh tissue was frozen in approximately 50-mg aliquots overnight. Ethanol (1 ml of 70%, per 50-mg aliquot) was added to the tissue, which was heated in a 60°C water bath for 6–7 h, until the tissue appeared free of chlorophyll. The ethanol was decanted off and the tissue washed in fresh 70% ethanol on a shaker. The ethanol was again decanted off and the tissue dried under vacuum. The residue remaining was the AIR containing the cell walls of the flowering stem.

2.2.4 Screening methods

2.2.4.1 Trifluoroacetic acid hydrolysis

The substrates for TFA hydrolysis were the AIR of *Arabidopsis* flowering stems, *Eucalyptus* pulp and *Eucalyptus* xylem. Each substrate was hydrolysed with 2 M TFA (10 mg of substrate per 1 ml TFA) at 120°C for 1 h. The hydrolysate was dried under vacuum to remove the TFA and resuspended in a volume of water equal to the original volume of TFA used.

For analysis by HPLC, the hydrolysis residue was washed twice in water. The washes and the hydrolysate were pooled before being dried

under vacuum to remove the TFA. The hydrolysates were resuspended in a volume of water equal to the original volume of TFA used and filtered through a 0.2-μm Gelman Supor Acrodisc filter. The hydrolysis residue was dried under vacuum and weighed.

2.2.4.2 Driselase digestion

The substrates for Driselase digestion were the AIR of *Arabidopsis* flowering stems, *Eucalyptus* pulp and *Eucalyptus* xylem. Each substrate was incubated with purified Driselase (10 mg of substrate per 250 μl 0.5% purified Driselase; Section 2.1.1) in pyridine/acetic acid/water (1:1:98, pH 4.7) at 37°C for 48 h. The digests were shaken for approximately 30 min prior to incubation at 37°C.

For analysis by HPLC, after incubation the digest was decanted off the cell wall residue, which was washed twice in a volume of water equal to the original volume of buffer used. The washes and digest were pooled and ethanol-precipitated to remove the protein (Section 2.1.3). The solution was dried under vacuum then resuspended in a volume of water equal to the original volume of Driselase in buffer used and filtered through a 0.2-μm Gelman Supor Acrodisc filter. The digestion residue was dried under vacuum and weighed.

2.2.5 Separation of screen products

2.2.5.1 Thin layer chromatography

The products of TFA hydrolysis (6 μl of each hydrolysate) were separated on cellulose TLC sheets backed with plastic (20 × 20 cm, layer thickness 0.1 mm, Merck). The solvent system used was butan-1-ol/acetic acid/water (3:1:1) for 9 h, followed by ethyl acetate/pyridine/water (10:4:3) for 4 h in the same dimension. A marker solution (3 μl of a 0.5% solution of each of galacturonic acid, glucuronic acid, galactose, glucose, mannose, arabinose, xylose, fucose, ribose and rhamnose, in 0.5% chlorobutanol) was

used. The cellulose TLCs were dried and stained with aniline hydrogen-phthalate (Section 2.2.7).

2.2.5.2 Paper chromatography

The products of Driselase digestion were separated by descending chromatography on Whatman No. 1 chromatography paper (46 × 57 cm). The solvent system used was ethyl acetate/ pyridine/ water (8:2:1, v/ v/ v) for 70 h. Prior to loading onto the paper chromatogram (PC), 30 µl of digest was mixed with 15 µl 30% formic acid to denature the Driselase. A marker solution (30 µl of a 0.5% solution of each of galacturonic acid, glucuronic acid, cellobiose, isoprimeverose, xylobiose, galactose, glucose, mannose, arabinose, xylose, fucose, ribose and rhamnose, in 0.5% chlorobutanol) was also loaded. Paper chromatograms were dried before being stained with aniline hydrogen-phthalate, Section 2.2.7.

2.2.5.3 HPLC protocols

Products of Driselase digestion and TFA hydrolysis were analysed on a Dionex HPLC with a CarboPac PA1 column. Sodium hydroxide (0.5 M, 0.6 ml/ min) was added post column, and a pulsed amperometric detector (PAD) was used.

Method 1 was used to separate the monosaccharide and uronic acid screen products. This method involved a sodium hydroxide gradient (0 mM to 800 mM NaOH, Figure 2.1) and took 85 minutes per sample.

Method 2 separated the disaccharide products of Driselase digestion, which were not fully separated by method 1. This method also involved a sodium hydroxide gradient but from 30 mM to 600 mM NaOH (Figure 2.2) with an isocratic gradient of 30 mM NaOH from t = 0 min to t = 42 min. This method took 61 minutes per sample.

Calibration

Samples were run on the HPLC as batches of up to 40 samples, with

calibration standards at the beginning of the batch. The concentration of the standards ranged from 0.1 mg/ ml (concentration of each component in the standard mix) to 0.02 mg/ ml. Standards of 0.05 mg/ ml were run between groups of samples to check that the calibration was accurate and that there was no drift in retention times. Chromatograms were calibrated using the external standards and Dionex software (AI-450, v3.2). For Driselase digests a Driselase only control was also ran, as Driselase undergoes some autolysis upon incubation. The concentration of the Driselase only products were subtracted from the samples before they were analysed.

2.2.6 Statistical analysis

Analysis of variance (anova, Rohlf and Sokal, 1981; Sokal and Rohlf, 1995) was used to compare the differences in product concentration between groups of replicates for Ws and Columbia (genetically homogenous lines). Where samples from different batches were to be compared, anova was used to show that there were no significant differences between the product concentrations of the wild type samples run in those batches. This was taken as evidence that it was valid to compare samples from the batches concerned. As the Feldmann lines were not genetically homogenous the Student's t distribution (Rohlf and Sokal, 1981; Sokal and Rohlf, 1995) was used to compare the differences between individual transformed plants and the group of Ws replicates.

Where a sample was run in two or more batches the mean concentration for the screen products separated was used in further analysis. For the Driselase digestion products, two different dilutions of the sample were used so accurate values for the most and least concentrated components (glucose and fucose, respectively) could be obtained. The concentrations of mid-range components were calculated from both dilutions of the sample, where the compound could be resolved using both HPLC methods.

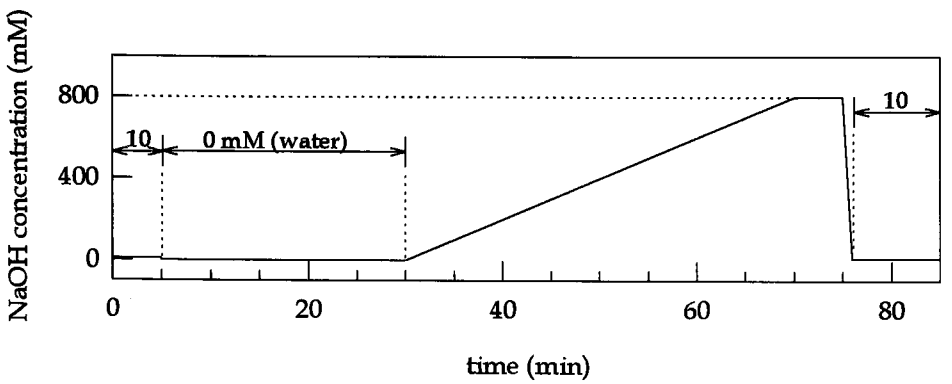

Figure 2.2 HPLC method 1. A sodium hydroxide gradient over 85 min (t = 0 to 4.9 min isocratic elution with 10 mM NaOH; t = 5 to 30 min, isocratic water; t = 30 to 70 min, linearly increasing NaOH concentration up to 800 mM; t = 70 to 75 min, 800 mM NaOH; t = 76 to 85 min, 10 mM NaOH). Eluent flow rate was 1 ml/ min throughout.

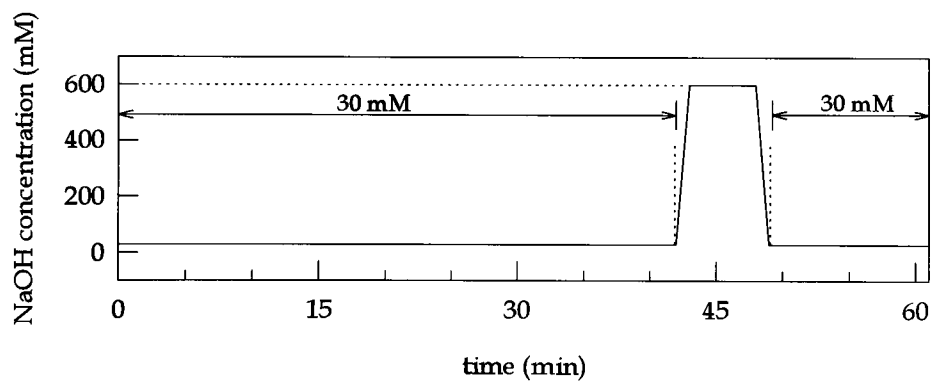

Figure 2.3 HPLC method 2. A sodium hydroxide gradient over 61 min (t = 0 to 42 min, isocratic 30 mM NaOH; t = 43 to 48 min, isocratic 600 mM NaOH; t = 49 to 61 min, isocratic 30 mM NaOH). Eluent flow rate was 1 ml/ min throughout.

2.2.7 Staining of chromatograms

Aniline hydrogen-phthalate (Fry, 1988) was used to stain monosaccharides (products of TFA hydrolysis and Driselase digestion) and reducing disaccharides (products of Driselase digestion) on PC and TLC. Hexoses stained brown, pentoses a pink/purple colour and uronic acids orange.

Immediately before use 100 ml stock (Table 2.5) was mixed with 0.5 ml aniline. The PC/ TLC was wetted with the solution, allowed to dry for 3–5 min in a fume hood, then heated for 5 min at 105°C. The detection limit was approximately 0.4 μg arabinose per spot on PC.

Table 2.5. Composition of stock solution for aniline hydrogen-phthlate stain.

Compound	per l stock
phthalic acid	16 g
acetone	490 ml
diethyl ether	490 ml
water	20 ml

2.2.8 Photography of chromatograms

Stained PCs and TLCs were photographed using Kodak Gold film, 200 ASA, under white light (f8 and 1/8 s exposure, with a "Cokin" chromofilter, blue (80B)) or 366-nm ultraviolet illumination (f5.6 and approximately 1 min exposure, with a Hoya yellow, 49m Y[K2] filter).

2.3 *Arabidopsis* genetics

2.3.1 Selfing of *Arabidopsis* plants

Plants were allowed to self-fertilise (outcrossing occurs at a frequency of less than 0.0001-0.01%; Feldmann and Marks, 1987; Feldmann *et al.*, 1989). To collect seeds, bags (available from Coutaulds Packing, Bristol) were placed over the whole stem before silique maturation. The bag and stem were allowed to hang down over the outside edge of the pot so that the seeds, when released, would fall to the bottom of the bag. Once all the siliques on a stem had matured, the stem was cut off the plant and left in the bag to dry. Seeds were collected by cutting off a corner of the bag and allowing the seeds to fall into a grease-proof paper envelope. The seeds were stored in these envelopes at room temperature until required.

2.3.2 Selection of kanamycin resistant plants

The T-DNA insert used in the Feldmann lines (Feldmann and Marks, 1987; Feldmann *et al.*, 1989) contains a kanamycin resistance gene. For selection of T-DNA tagged lines, seeds (surface-sterilised, Section 2.2.2.2) were sown on solid medium plates containing kanamycin (Table 2.6). The agar/ sucrose solution was autoclaved prior to the addition of kanamycin monosulphate (filter-sterilised through a 0.2-μm Gelman Supor Acrodisc filter). After 4 to 5 days the seeds germinated and after a further 2 days the cotyledons of kanamycin sensitive lines had been bleached, while kanamycin resistant seedlings appeared normal. At 2 weeks post-germination, the seedlings were transferred to plates without kanamycin.

Table 2.6 Composition of agar plates for selection of kanamycin resistant seedlings

compound	amount per l medium
agar	8 g
sucrose	10 g
ATS nutrient solution	900 ml
1% kanamycin monosulphate in ATS [final concentration = 0.1%]	100 ml

3 Analysis of *Eucalyptus* Wood and Pulp

The aim of this section was to establish the polysaccharides present in the wood pulp used in paper production and also the levels of these polysaccharides in the xylem of *Eucalyptus* spp. The presence of non-cellulosic polysaccharides in pulp can affect the quality of some end-products (e.g acetates) and repeated treatments to remove these contaminants can degrade the cellulose microfibrils and hence the quality of the pulp.

3.1 Xylem

E. globulus and *E. grandis* (Section 2.2.1.1) were grown in a greenhouse (Section 2.2.2.1). Stems (approximately 1 year old) were freeze-dried and stripped of the cork and phloem layers. Any phloem remaining would make up a only a small proportion of the tissue. The xylem was delignified (Section 2.1.5) and then either hydrolysed with TFA (Section 2.2.4.1) or digested with purified Driselase (Sections 2.1.1 and 2.2.4.2). The screen products were separated on a Dionex HPLC using methods 1 and 2 (Section 2.2.5.3).

3.1.1 Delignification of *Eucalyptus* xylem

The xylem from stems of *E. grandis* and *E. globulus* was delignified using the method of Whistler and BeMiller (1963; Section 2.1.4). The residues were dried and weighed. The mass extracted by this method was 185.1 mg/g xylem for *E. grandis* and 165.7 mg/g xylem for *E. globulus* (Table 3.1). This method does not allow a quantitative determination of lignin content; however, the majority of the mass lost will be due to lignin extraction. The lignin content of *Eucalyptus* spp. wood is reported to be 23% of the extractive free wood (Timmell, 1965) which would imply that not all of the lignin was extracted from the wood using this method.

Table 3.1 The mass of the delignified residue of *E. grandis* and *E. globulus*. Dry xylem was delignified (Section 2.1.4) and the residue dried and weighed.

Species	Mass of delignification residue (mg/ g xylem)	% xylem extracted
E. grandis	814.9	18.5
E.globulus	834.3	16.6

3.1.2 TFA hydrolysis

Delignified xylem of *E. grandis* and *E. globulus* (Section 2.1.4) was hydrolysed with TFA (Section 2.2.4.1) and the hydrolysis products were separated on a Dionex HPLC (Section 2.2.5.3) using method 1 (Figure 2.1).

3.1.2.1 Hydrolysis residue

There was no significant difference between *E. grandis* and *E. globulus* in the mass of the hydrolysis residues (Table 3.2), which were approximately 40% of the mass of the delignified xylem. This result indicates that the proportion of TFA-susceptible polymers in the delignified xylem was approximately the same for the two species.

3.1.2.2 Total mass of identified carbohydrate products

The total mass of identified hydrolysis products from *E. globulus* was 10% less than from *E. grandis,* although this difference was not significant (Table 3.2). This implies that the proportion of TFA-susceptible polysaccharides in the delignified xylem was approximately the same for the two species.

3.1.2.3 Individual hydrolysis products

The major components of the hydrolysates were glucose (27–33 mol%) and xylose (41–44 mol%). The xylose was probably derived primarily from

xylan and the glucose from the non-cellulosic cell wall polysaccharides (glucomannan, xyloglucan and a small amount from pectin; Darvill *et al.*, 1978) and starch. Although, it has been reported previously that TFA can hydrolyse a small proportion of the cellulose from cell walls (5–10%; Selvendran and Ryden, 1990).

There were significant differences between *E. grandis* and *E. globulus* in the composition of their hydrolysates (Table 3.2). *E. globulus* had significantly lower proportions of fucose (50% of the *E. grandis* mol% mean) and glucose (84%), but significantly more arabinose (175%) and mannose (118%). The differences between *E. globulus* and *E. grandis* in the concentrations of glucose and arabinose can be easily seen in Figure 3.1.

The level of fucose was extremely low in both species and, while there was a significant difference between the species, the identity of the corresponding HPLC peaks can be regarded as uncertain (Table 3.2 and Figure 3.1).

There were 27–33 mol% of glucose in the hydrolysates, indicating that starch and non-cellulosic cell wall polysaccharides (glucomannan, xyloglucan and possibly callose) together make up a significant proportion of the xylem. The higher level of glucose in the *E. grandis* hydrolysate could indicate an increase in any of these. Glucomannan has an approximate ratio of glucose to mannose of 1:2 (Timell, 1965). Therefore, from the mannose data, the amount of glucose derived from glucomannan would only be 5.0% of the glucose for *E. grandis* and 7.6% for *E. globulus*. Xyloglucan is unlikely to be the cause of the large amount of glucose as it is only a minor component of wood. Therefore the difference in glucose levels between the two species could be due to increased starch in the ray cells of *E. grandis*.

The concentration of mannose in the TFA hydrolysates cannot be calculated with certainty as in this case the resolution of xylose and mannose on the HPLC (e.g. Figure 3.1) was not enough to give reliable mannose data, because of the relatively high concentration of xylose. However, using the data obtained, it can be suggested that while there was a significant

difference between *E. grandis* and *E. globulus* in the proportion of mannose (mol%), the concentration (μmol/ g delignified xylem, dx) was not significantly different. The significant difference between the two species in the proportions of mannose was due to the higher concentration of glucose in *E. grandis* than in *E. globulus*. Therefore the levels of glucomannan are probably similar in the wood of *E. grandis* and *E. globulus*. The levels of xylose were also similar in the two species, indicating that there was no difference between the two species in the amount of xylan present. The uncertainty in the mannose concentration will also affect the xylose data but to a much lesser extent as the concentration of xylose was 11–13 × that of mannose.

The increased arabinose in *E. globulus* could be due to increased glycoproteins, increased arabinosylation of xylans, or to increased arabinogalactans in the pectic component.

The galacturonic acid in the hydrolysate is derived from the pectic polysaccharides. However, the concentration in the hydrolysate is unlikely to indicate quantitatively the level of pectins present in the wood, as uronic acid glycosyl bonds are relatively resistant to the hydrolysis conditions used. Also, Imai and Terashima (1992a) found that quantitative analysis of the galacturonans in wood tissue was not possible owing to strong binding to lignin and hemicellulose.

Table 3.2 The TFA hydrolysis products of the delignified xylem of *Eucalyptus* spp. The delignified cell walls were hydrolysed with 2 M TFA (Section 2.2.4.1). Hydrolysis products were separated on a Dionex HPLC (Section 2.2.5.3). Chromatograms were calibrated using external standards and Dionex software. The figures shown are means, with standard deviations in brackets; figures in bold are µmol/ g delignified xylem. n = number of replicate hydrolysates of one preparation of delignified xylem; dx = delignified xylem; P = probability that the mol% values come from the populations with the same mean (as calulated by the anova method, Section 2.2.6);.

Eucalytpus species	Products of TFA hydrolysis [mol%; (standard deviation); µmol/ g dx]									Total mass of identified carbohydrate products (mg/ g dx)	Mass of hydrolysis residue (mg/ g dx)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA		
E. grandis	0.2	2.6	2.8	6.3	32.6	41.6	3.3	6.3	4.5	333	402
n = 5	(0.0)	(0.1)	(0.2)	(0.8)	(3.2)	(4.5)	(0.5)	(1.9)	(1.5)	(42.2)	(37.9)
	3.5	**50.9**	**56.0**	**123**	**646**	**833**	**64.9**	**123**	**86.2**		
E. globulus	0.1	2.6	3.5	5.6	27.3	43.8	3.9	8.3	4.9	300	391
n = 5	(0.1)	(0.2)	(0.2)	(0.3)	(3.2)	(2.1)	(0.4)	(2.1)	(1.1)	(29.2)	(40.0)
	2.1	**46.3**	**62.9**	**99.9**	**458**	**788**	**69.9**	**151**	**88.4**		
P =	**< 0.10**	**> 0.75**	**< 0.001**	**> 0.10**	**< 0.05**	**> 0.25**	**< 0.10**	**> 0.10**	**> 0.50**	**> 0.10**	**> 0.50**

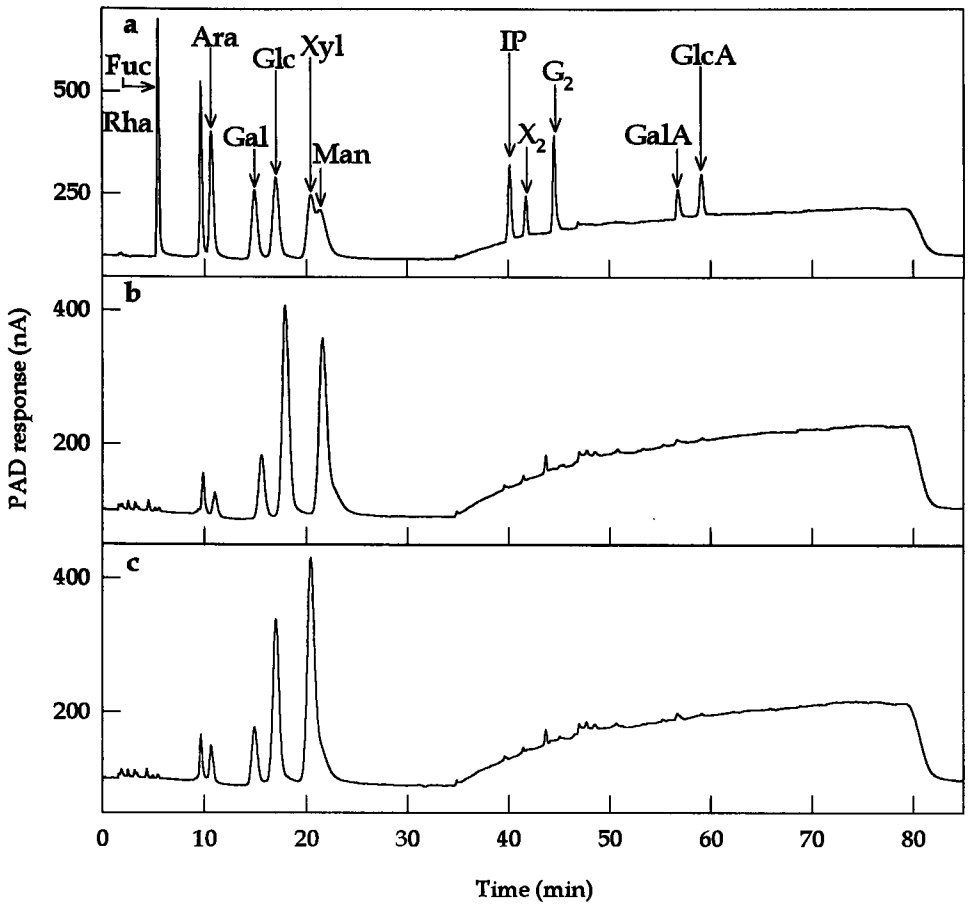

Figure 3.1 TFA hydrolysis products of the delignified xylem of *E. globulus* and *E. grandis* stems. Delignified *Eucalyptus* xylem (Section 2.1.5) was hydrolysed with 2 M TFA (Section 2.2.4.1). The hydrolysis products were separated on a Dionex HPLC using method 1 (Section 2.2.5.3). **(a)** A mix of standards, each at a concentration of 0.05 mg/ ml; the TFA hydrolysis products of **(b)** *E. grandis* delignified xylem and **(c)** *E. globulus* delignified xylem. One in five dilutions of the original hydrolysates are shown.

3.1.3 Driselase digestion

Delignified xylem of *E. grandis* and *E. globulus* (Section 2.1.5) was incubated with Driselase (Sections 2.1.1 and 2.2.4.2) and the digestion products were separated on a Dionex HPLC (Section 2.2.5.3) using method 1 (Figure 2.1) and method 2 (Figure 2.2). Method 1 separated the monosaccharide and uronic acid products, while method 2 separated the disaccharide components of the digest. As Driselase can undergo slight autolysis upon incubation, a Driselase-only control was also analysed by HPLC (Figures 3.2d and 3.3d). The values shown for the *Eucalyptus* screen products (Table 3.3) have had the concentrations of the Driselase-only products subtracted from them.

3.1.3.1 Digestion residue

There was no significant difference between the mass of the digestion residues of *E. grandis* and *E. globulus* (Table 3.3). Both species had residues that were approximately 67–68% of the original mass of the delignified xylem. This implies that for both species the Driselase-susceptible polymers made up the same proportion of the delignified xylem.

3.1.3.2 Total mass of identified products

The total mass of identified carbohydrate products from the Driselase digestion of *E. grandis* was significantly lower (by 22%) than in *E. globulus* (Table 3.3). This indicates that the proportion of Driselase-susceptible polysaccharides in the delignified xylem was lower in *E. grandis* than in *E. globulus*.

3.1.3.3 Identified products

The major products in the digests were glucose (derived from cellulose, starch and non-cellulosic wall polysaccharides) and xylobiose (derived from xylan).

There were significant differences between *E. grandis* and *E. globulus* in the compositions of the Driselase digests (Table 3.3). *E. globulus* had significantly increased levels of fucose (200% of the *E. grandis* mol% mean), arabinose (117%), xylose (153%), mannose (117%) and xylobiose (155%) and significantly decreased levels of rhamnose (56%), galactose (63%) and glucose (83%). The differences between *E. globulus* and *E. grandis* in the concentrations of arabinose, galactose and xylobiose can be seen in Figure 3.2 (galactose) and Figure 3.3 (arabinose and xylobiose).

The level of fucose was extremely low in both species and, while there was a significant difference between the species the concentration in *E. globulus* was only increased by 1.0 μmol/ g dx (Table 3.3).

The difference in the proportion of some of the products (glucose and rhamnose) in the digests was due primarily to the increased concentrations of xylobiose and xylose in *E. globulus* which reduced the percentage that other sugars made up of the total identified carbohydrate products. The actual concentrations of these sugars in the digests (μmol/ g dx) were not significantly different.

Xyloglucan is present in wood tissue at very low levels, as can be seen by the low proportion of isoprimeverose in the digest (0.5 mol%). There was no significant difference between the two species in the proportion of the identified products that was isoprimeverose. However, again this was due to the higher concentration of xylobiose and xylose in *E. globulus*. The actual concentrations of isoprimeverose (μmol/ g dx) were significantly different. This difference in the concentration of isoprimeverose could indicate that *E. grandis* has decreased levels of Driselase-susceptible xyloglucan.

The levels of both xylobiose and xylose were higher in *E. globulus* than in *E. grandis* whether expressed as mol% or μmol/ g AIR. As xylobiose is indicative of xylan, this implies that *E. globulus* has higher levels of Driselase-susceptible xylan in the delignified xylem than *E. grandis*. The free xylose in the Driselase digest was presumably also derived primarily from

xylan.

There was significantly more mannose in *E. globulus* (mol%) than in *E. grandis*. The difference was very small; however, the proportion of mannose in *E. grandis* is reduced by the increase in xylobiose and xylose so that the difference between the two species in the concentration of mannose is significant ($P < 0.05$). This indicates that *E. globulus* has more Driselase-susceptible, mannose-containing polysaccharides (glucomannans) than *E. grandis*.

The concentration of mannose in the Driselase digests cannot be calculated with certainty as in this case the resolution of xylose and mannose on the HPLC (e.g. Figure 3.2) was not enough to give reliable mannose data, owing to the relatively large high concentration of xylose.

While the proportions of the digests that were galacturonic acid were not significantly different for the two species, the concentration of galacturonic acid (μmol/ g dx) in the digest of *E.globulus* was higher by 30% than in that of *E. grandis*. This was because of the increased levels of xylose and xylobiose in the digest of *E. globulus*. The level of rhamnose was significantly lower in *E. globulus* than in *E. grandis*. These results indicate that there was more Driselase-susceptible galacturonic acid in *E. globulus* than in *E. grandis*, but less Driselase-susceptible rhamnose.

The increased level of arabinose in *E. globulus* could be due to increased glycoproteins, increased arabinosylation of xylans or increased arabinogalactans in the pectic component. Galactose was lower in *E. globulus* (by 37%) than in *E. grandis*. Galactose is present in xyloglucan, rhamnogalacturonans and arabinogalactans in the pectic component and the carbohydrate component of glycoproteins.

Table 3.3 The Driselase digestion products of the delignified xylem of *Eucalyptus* spp. The delignified cell walls were digested with Driselase (Section 2.2.4.2). Digestion products were separated as described for Table 3.2. The figures shown are means, with standard deviations in brackets; figures in bold are µmol/ g delignified xylem. n = number of replicate digests of one preparation of delignified xylem; dx = delignified xylem; n.a. = not applicable; P = probability that the mol% values come from the populations with the same mean (as calulated by the anova method, Section 2.2.6); n.a. = not applicable. The products of the Driselase-only controls are given in µmol/ 25 ml 0.5% Driselase, which is equivalent to µmol/ g dx; the values for the control products have been subtracted from the screen products for *Eucalyptus* shown below.

Eucalytpus species	Products of Driselase digestion [mol%; (standard deviation); µmol/ g dx]										**Total mass of identified carbohydrate products (mg/ g dx)**	**Mass of hydrolysis residue (mg/ g dx)**
	Fuc	**Rha**	**Ara**	**Gal**	**Glc**	**Xyl**	**Man**	**XG_2**	**X_2**	**GalA**		
E. grandis	0.1	0.9	1.8	6.4	61.1	9.5	0.6	0.5	13.7	5.3	166	673
n = 5	(0.0)	(0.1)	(0.2)	(0.6)	(3.0)	(0.3)	(0.1)	(0.1)	(3.0)	(0.6)	(41.5)	(121)
	0.9	**7.7**	**15.4**	**56.6**	**535**	**82.7**	**5.4**	**4.0**	**114**	**44.7**		
E. globulus	0.2	0.5	2.1	4.0	50.9	14.5	0.7	0.5	21.2	5.5	212	684
n = 6	(0.0)	(0.2)	(0.1)	(0.4)	(1.3)	(0.6)	(0.1)	(0.1)	(1.4)	(0.3)	(28.4)	(153)
	1.9	**5.6**	**22.5**	**42.8**	**547**	**155**	**7.8**	**5.3**	**226**	**58.3**		
P =	**< 0.001**	**< 0.005**	**< 0.005**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.10**	**> 0.75**	**< 0.001**	**> 0.50**	**< 0.10**	**> 0.75**
	µmol/ 25 ml 0.5% Driselase											
Driselase-only control	0.0	1.0	0.6	0.1	0.5	0.0	3.1	0.0	2.5	7.4	3.1	
n = 6	(0.0)	(0.1)	(0.05)	(0.04)	(0.1)	(0.0)	(0.4)	(0.0)	(0.2)	(11.0)	(2.1)	n.a.

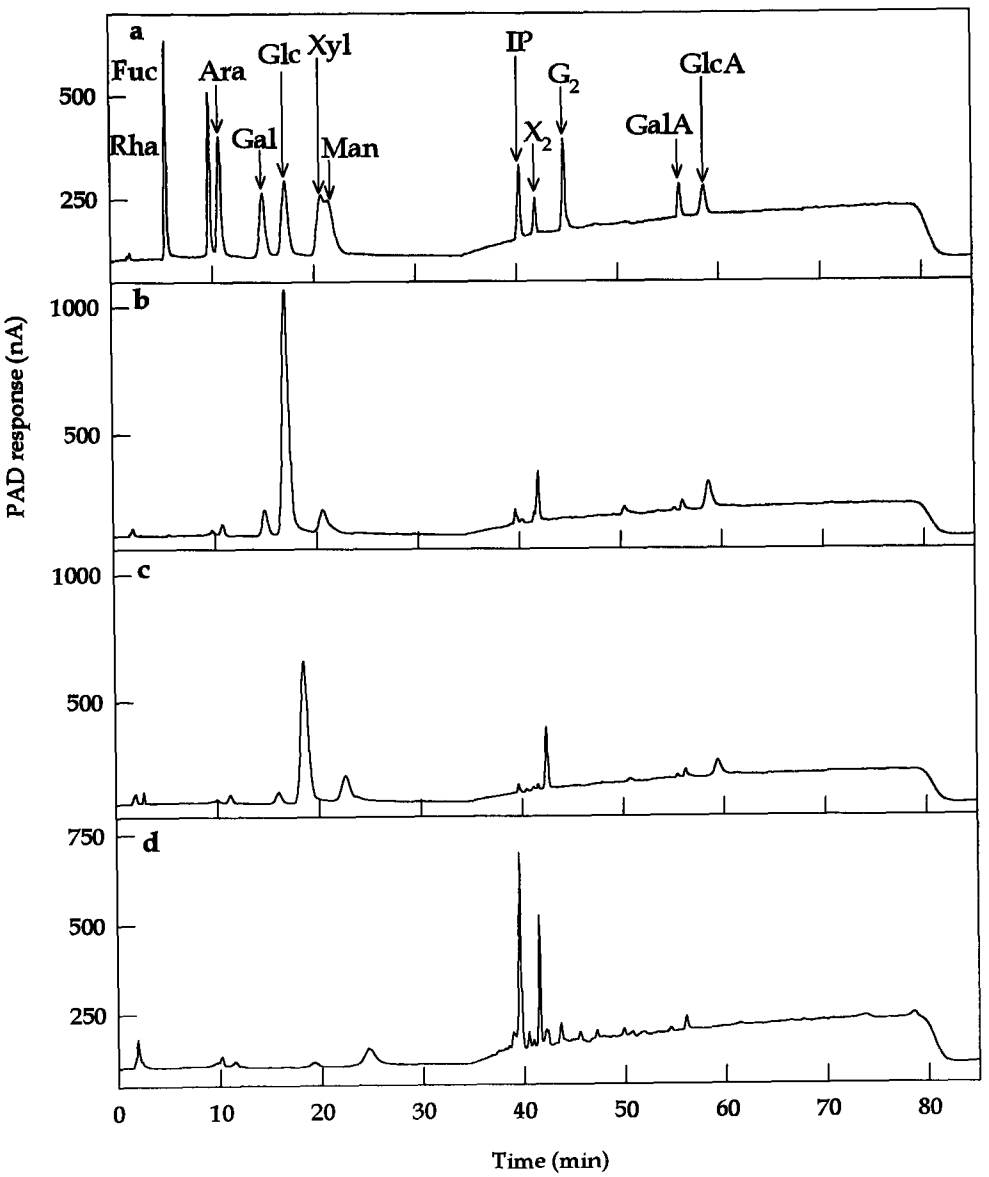

Figure 3.2 Driselase digestion products of the delignified xylem of *E. globulus* and *E. grandis* stems. Delignified *Eucalyptus* xylem (Section 2.1.5) was incubated with 0.5% Driselase (Section 2.2.4.2). The digestion products were separated on a Dionex HPLC using method 1 (Section 2.2.5.3). **(a)** A mix of standards, each at a concentration of 0.05 mg/ ml; the Driselase digestion products of **(b)** *E. grandis* delignified xylem (a one in twenty dilution of the original digest is shown) and **(c)** *E. globulus* delignified xylem (one in twenty dilution of the original digest); **(d)** a Driselase-only control (undiluted digest is shown).

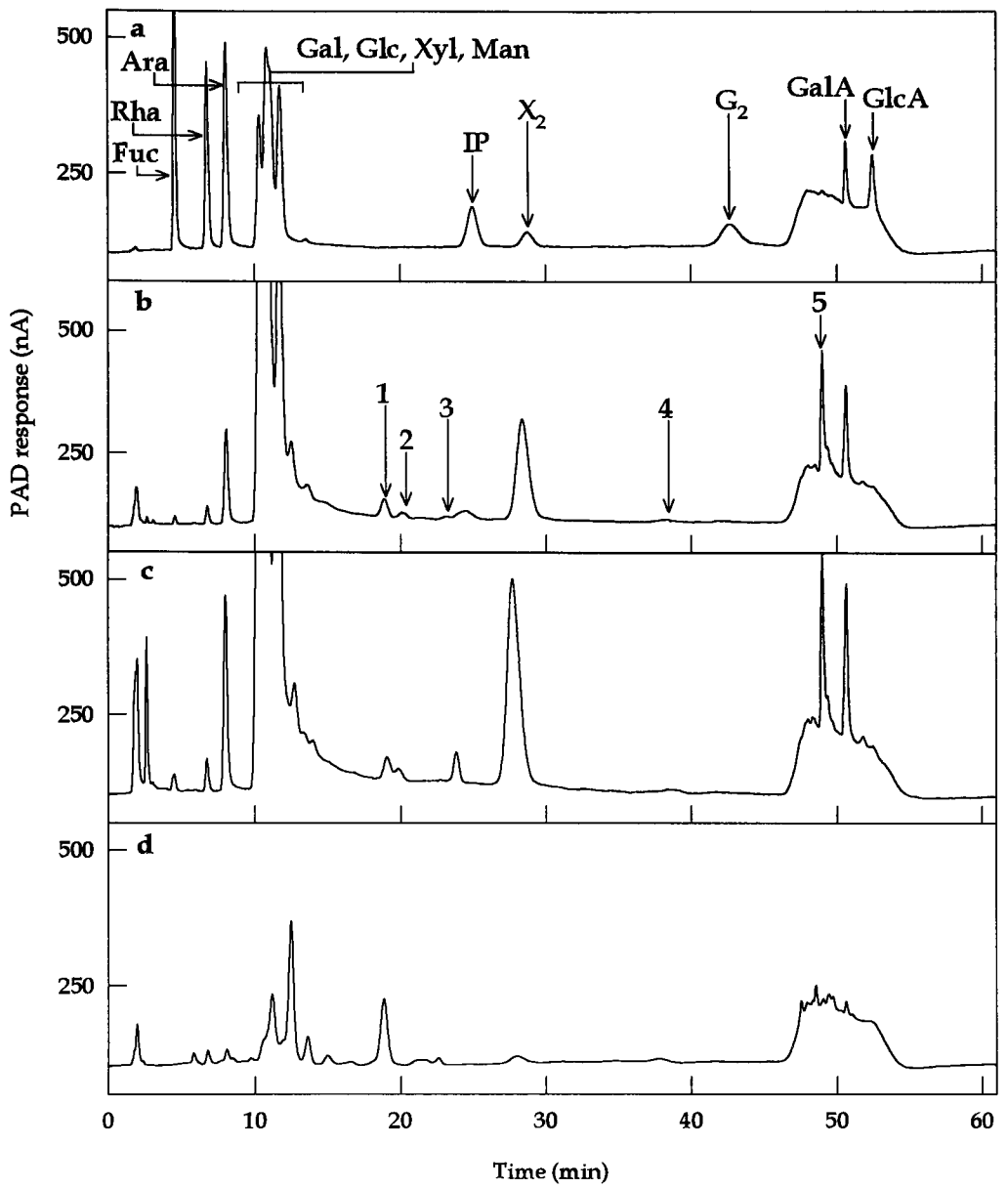

Figure 3.3 Driselase digestion products of the delignified xylem of *E. globulus* and *E. grandis* stems. Details as for Figure 3.2 except that HPLC method 2 was used and **(b)** and **(c)** show 4-fold dilutions; 1–5 = unidentified products (Section 3.1.3.4).

3.1.3.4 Unidentified products

A number of products of the Driselase digestion were not identified (Peaks 1–5, Figure 3.3). The areas of these peaks as a percentage of the total chromatogram area are shown in Table 3.4. The corresponding values for isoprimeverose and galacturonic acid are shown for comparison. There were significant differences between *E. grandis* and *E. globulus* in the percentage area of peaks 1, 2 and 5. However, the total percentage areas of peaks 1 to 4 are only 72% (for *E. grandis*) and 85% (for *E. globulus*) of the percentage area of the isoprimeverose peak, which accounts for only 0.5 mol% of the identified products (Table 3.3). Therefore, these products can be considered to be minor and were not investigated further. Peak 5 eluted in the region of the uronic acids, and was 141% (for *E. grandis*) and 92% (for *E. globulus*) of the percentage area of the galacturonic acid peak. From its shape (Figure 3.3) peak 5 appears to be composed of more than one compound. Peak 5 makes up a significantly (30%) larger proportion of the total chromatogram area in *E. grandis*, than in *E. globulus*.

Glucuronic acid was not detected in the Driselase digests of either *E. globulus* or *E. grandis* while it was present in the TFA hydrolysates of both species (Table 3.2). Glucuronic acid is present in wood cell walls primarily as a substituent of glucuronoxylan, linked by an α-(1→2) or α-(1→3) glycosidic bond to the β-(1→4)-linked xylan backbone. Driselase is not known to contain an α-D-glucuronidase, while it does contain β-(1→4)-D-xylanase and β-D-xylosidase activities. Thus the glucuronoxylan of wood cell walls would be broken down into xylose, xylobiose and probably glucuronosyl-xylose and/or glucuronosyl-xylosyl-xylose. The glucuronic acid in the xylan of wood cell walls is known to be methylated (Hazlewood and Gilbert, 1993) so a methylated glucuronosyl-xylo(bio)se would also be expected to be present. This could account for the decreased yields of xylose and xylobiose in *E. globulus* compared to *E. grandis* (Section 3.1.3.3) and could also indicate an increased level of substitution by glucuronic acid in the xylan of *E. grandis*.

Table 3.4 The unidentified Driselase digestion products of *Eucalyptus* spp. delignified xylem. The values for IP and GalA are shown for comparison. The values shown are means; n = number of replicate digests of one preparation of delignified xylem; P = probability that the values come from populations with the same mean (as calculated by the anova method, Section 2.2.6).

	% of total chromatogram area							Total % area
Species	**Peak 1**	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**	**IP**	**GalA**	**for Peaks 1-4**
E. grandis **n = 5**	0.4	0.9	0.1	0.4	9.9	2.5	7.0	1.8
E. globulus **n = 6**	0.1	1.4	0.3	0.5	7.6	2.7	8.3	2.3
P =	< 0.05	< 0.001	> 0.10	> 0.25	< 0.001	-	-	

3.1.4 Discussion

In both species the TFA residue was lighter than the Driselase residue, despite the fact that TFA cannot hydrolyse the majority of cellulose because of its crystalline structure (Selvendran and Ryden, 1990). It is possible that not all of the lignin was extracted from the xylem (Section 3.1.1) and the remaining lignin in some way interfered with the solubilisation of some of the cell wall polysaccharides by Driselase.

The Driselase and TFA residues of *E. grandis* were not significantly different in mass from those of *E. globulus*, while the total mass of TFA-solubilised products was approximately the same for the two species, the total mass of Driselase-solubilised identified products was significantly lower (by 22%) in *E. grandis* than in *E. globulus*. Therefore, the proportion of the delignified xylem that was Driselase-susceptible was the same in the two species but in *E. grandis* a lower proportion of the xylem was broken down to identified products. This could indicate an increased level in *E. grandis* of the unidentified peaks or another component of xylem such as wall-bound proteins or the unidentified products (Section 3.1.3.4).

The total mass of identified products was higher in the TFA hydrolysates (by 101% for *E. grandis*, and 41% for *E. globulus*) than in the Driselase digests. Again, this was suprising as TFA cannot hydrolyse the majority of cellulose (Selvendran and Ryden, 1990). The additional mass was primarily xylose although the concentrations of all the components of the TFA hydrolysate (except glucuronic acid which was not present in the Driselase products) were higher than in the Driselase digest. It is possible that the difference in xylose between the two screens was at least partly due to xylose being present in the Driselase digest as an unidentified product (e.g. a di/trisaccharide of glucuronic acid and xylose; Section 3.1.3.4).

3.1.4.1 Glucose

The difference between the TFA hydrolysis and Driselase digest levels of glucose was not significant for either species despite the fact that TFA (unlike Driselase) cannot hydrolyse the majority of cellulose (Selvendran and Ryden, 1990). This could indicate very low levels of cellulose digestion by Driselase or lower solubilisation of one of the other glucose-containing polysaccharides (such as starch) by Driselase than by TFA. If it is assumed that no cellulose is broken down by TFA hydrolysis and starch has a very low susceptibility to Driselase digestion, then the glucose in the Driselase products will primarily be derived from cellulose and xyloglucan, while the glucose in the TFA products will be derived from starch and xyloglucan. Using these assumptions the approximate yields of glucose derived from each of the glucose-containing polysaccharides can be calculated (Table 3.5).

The literature value for the level of cellulose in the xylem of hardwoods is approximately 20% (Timell, 1965) which in this case upon complete hydrolysis would yield approximately 1235 μmol glucose/ g dx. The yields of glucose in the Driselase digests were 535 and 547 μmol/ g dx for *E. grandis* and *E. globulus*, respectively (Table 3.3). This

indicates either that less than half the cellulose in the wood was digested by Driselase, possibly owing to the presence of residual lignin, or that the susceptibility of one or more of the other glucose-containing polysaccharides to Driselase is less than TFA. It is known that starch is only very slightly hydrolysed to glucose by Driselase unlike TFA (S. Aldington, pers. commun.).

These results indicate that *E. grandis* and *E. globulus* have approximately the same levels of Driselase-susceptible cellulose, but *E. grandis* had 42% more starch-derived glucose and 25% less xyloglucan-derived glucose than *E. globulus*.

Table 3.5 The calculated yields of glucose derived from cellulose, starch and xyloglucan. **1** = yield of glucose in the Driselase digest; **2** = yield of isoprimeverose in the Driselase digest; **3** = yield of glucose in the TFA hydrolysate; **4** = glucose derived from cellulose (= $Glc_D - \frac{1}{3}Glc_{IP}$); **5** = glucose present in xyloglucan (= $\frac{4}{3}Glc_{IP}$); **6** = glucose derived primarily from starch (= $Glc_T - Glc_{XG}$).

Species	Glc_D [1] (µmol/ g dx)	Glc_{IP} [2] (µmol/ g dx)	Glc_T [3] (µmol/ g dx)	Glc_C [4] (µmol/ g dx)	Glc_{XG} [5] (µmol/ g dx)	Glc_S [6] (µmol/ g dx)
E. grandis	535	4.0	646	534	5.3	641
E. globulus	547	5.3	458	545	7.1	451

3.1.4.2 Xylose

The percentage of the total xylose in the Driselase digest that was due to isoprimeverose was 0.7% for *E. grandis* and 1.0% for *E. globulus*, so it can be assumed that the majority of the xylose in the TFA hydrolysate was derived from xylan. *E. grandis* had more xyloglucan than *E. globulus*, although only by 1.3 µmol isoprimeverose/ g dx.

Assuming that all the xylose present in the AIR was solubilised by TFA, the difference between the level of xylose in the TFA hydrolysate and the total amount of xylose present in the Driselase screen (as xylose, xylobiose and isoprimeverose) is the amount of Driselase-resistant xylose. The total level of xylose (Xyl_T, Table 3.6) was not significantly different between the two species; however, Driselase digested 78% of the total xylose in *E. globulus* but only 38% in *E. grandis* (Table 3.6). These differences could be due to a higher degree of acetylation in the xylan of *E. grandis* compared with *E. globulus* as acetylated β-xylose residues are resistant to Driselase (Fry, 1988). A proportion of the 'Driselase-resistant' xylose may be present as one or more of the unidentified Driselase-products (Section 3.1.3.4).

Table 3.6 Driselase-resistance of xylose in two Eucalypt species. 1 = yield of xylose in TFA hydrolysate; **2** = total yield of xylose in the Driselase digest (including xylose present in the form of xylobiose and isoprimeverose); **3** = Driselase-resistant xylose (= $Xyl_T - Xyl_D$).

Species	Xyl_T [1] (μmol/ g dx)	Xyl_D [2] (μmol/ g dx)	Xyl_{DR} [3] (μmol/ g dx)	Xyl_{DR} as a % of Xyl_T
E. grandis	833	314	519	62.3
E. globulus	788	612	176	22.3

3.1.4.3 Mannose

The levels of mannose in the TFA hydrolysates imply that the amount of glucomannans in the xylem cell walls of the two species was approximately the same, while from the Driselase digests it seems that *E. globulus* had slightly higher levels than *E. grandis*. However, the concentration of mannose in the Driselase digests can not be calculated with certainty as in this case the resolution of xylose and mannose on the HPLC

(e.g. Figure 3.2) was not enough to give reliable mannose data, owing to the relatively high amount of xylose.

3.1.4.4 Galacturonic acid and rhamnose

There was no difference between the two species in the galacturonic acid values for the Driselase or TFA screens. However, in both species the concentration of galacturonic acid in the TFA products was approximately twice that found in the Driselase products. This was suprising as galacturonic glycosyl bonds are relatively resistant to the TFA hydrolysis conditions used. Some of the galacturonic acid in the AIR could be present in a pectic polymer that is Driselase-resistant (e.g. acetylated galacturonic acid, P. Perrone, pers. commun.; galacturonic acid in rhamnogalacturonan-II, S. Aldington, pers. commun.).

The significantly higher levels of rhamnose, seen in the Driselase products of *E. globulus* compared to *E. grandis*, were not seen in the products of TFA hydrolysis. However, the levels of rhamnose found in the TFA hydrolysates were approximately 7 times those found in the Driselase digests. Again this could indicate that a proportion of the rhamnose in the AIR is present in a Driselase-resistant pectic polymer.

3.1.4.5 Arabinose

The yield of arabinose was significantly higher in *E. globulus* than in *E. grandis*, in both the Driselase and TFA screens. This could be because of increased arabinose in arabinogalactans, increased glycoproteins or increased arabinosylation of xylans.

3.1.4.6 Galactose

There was a significantly higher level of galactose in the Driselase products of *E. globulus*, compared with *E. grandis*. However, this difference was not seen in the TFA screen products. There was approximately twice the amount of galactose in the TFA screen products as was found in the

products of Driselase digestion. Again, this could be explained by a proportion of the galactose in the AIR being present in a Driselase-resistant form.

3.1.4.7 Glucuronic acid

Glucuronic acid was present in the TFA hydrolysate but was not detected in the Driselase digest. As there is no known α-D-glucuronidase activity in Driselase it is possible that glucuronic acid was present in the Driselase products as a glucuronosyl-xylose disaccharide and/or a glucuronosyl-xylosyl-xylose trisaccharide. These would both be derived from glucuronoxylan, which is a major component of secondary cell walls (James *et al.*, 1985). An unidentified product (Peak 5, Figure 3.3) of Driselase digestion eluted in the uronic acid region of the chromatogram, before galacturonic acid (Section 3.1.3.4). It is possible that this peak was at least partly due to the glucuronosyl-xylo(bio)se di/trisaccharide, and that the greater area of the peak in *E. grandis*, compared with *E. globulus*, indicates an increased level of substitution by glucuronic acid in the xylan of *E. grandis*. The major xylan in wood is known to be 4-*O*-methylglucuronoxylan (Hazlewood and Gilbert, 1993) so 4-*O*-methylglucurono-xylo(bio)se would also be expected to be present in the digest products. This could also account for the high 'Driselase-resistance' of the xylan of *E. grandis*, compared to *E. globulus* (Table 3.6). 4-*O*-Methylglucuronic acid would also be expected to be present in the TFA hydrolysate, but no marker for this methylated monosaccharide was available so its presence in the TFA digests could not be checked.

3.1.4.8 Summary

There was a greater mass of identified products in the Driselase digest of *E. globulus* than in that of *E. grandis*. This feature was correlated with a lower level of Driselase-resistant xylose and a lower proportion of unidentified products in *E. globulus* than in *E. grandis*. As there was no

difference between the two species in the level of total xylose (Xyl_T, Table 3.6), the difference in Driselase-resistant xylose (which includes any xylose present as an unidentified product) indicates a greater proportion of acetylated xylose residues and substitution with glucuronic acid in the xylan of *E. grandis,* than in that of *E. globulus.* The unidentified products may include (methyl)glucuronosyl-xylo(bio)se, which would not be digested by Driselase.

E. grandis wood was found to have less starch-derived glucose and a slightly lower level of xyloglucan than *E. globulus.*

The results indicate that a proportion of the pectic polysaccharides are resistant to Driselase digestion. *E. globulus* appears to have less Driselase-resistant rhamnose, galacturonic acid and galactose residues than *E. grandis.* The increased arabinose in *E. globulus* could be due to increased arabinogalactans, glycoproteins or increased arabinosylation of xylans.

The major wall polysaccharides in the xylem cell walls of hardwoods are reported to be cellulose, an acetylated (methyl)glucuronoxylan and glucomannan (Timmell, 1965); a small amount of pectin is also present (Imai and Terashima, 1992a). From my results it can be calculated that 10–11% (by mass) of the delignified xylem is due to the xylan backbone and 1.6–1.7% is due to glucomannans. The percentage of xylan backbone present in the delignified xylem is lower than expected however, it is possible that the delignification procedure extracted a proportion of the acidic xylan. It is not possible to calculate a percentage mass for cellulose owing to its incomplete solubilisation by Driselase. Further studies of cellulose could have been undertaken by H_2SO_4 hydrolysis using the method of Saeman (1945), but owing to time constraints this was not undertaken.

Xylan is a major component of *Eucalyptus* wood and as such could be a contaminant of cellulose pulps. This would interfere with the quality of the pulps produced and would require the use of extra extraction steps to remove it. Wood that had a lower proportion of xylan or had xylan that was more easily extracted would be of benefit to the pulp and paper industries.

3.2 Pulp

Card produced from *Eucalyptus* spp. was stirred in water to form a pulp suspension (Section 2.2.3.1). Aliquots of the suspension were either hydrolysed with TFA (Section 2.2.4.2) or incubated with purified Driselase (Sections 2.1.1 and 2.2.4.2). The products of the two screens were separated on a Dionex HPLC (Section 2.2.5.3) using HPLC method 1 (Figure 2.1).

3.2.1 TFA hydrolysis

The residue remaining after TFA hydrolysis of the pulp was approximately 90% of the original mass of the pulp (Table 3.7).

The products of hydrolysis were primarily xylose, glucose, galactose and galacturonic acid, with trace amounts (less than 1 mol% of the hydrolysate products) of rhamnose, arabinose and glucuronic acid (Table 3.7 and Figure 3.4). TFA cannot hydrolyse cellulose because of its crystalline structure. However it has been reported that TFA can hydrolyse a small proportion of the cellulose from cell walls (5–10%; Selvendran and Ryden, 1990). The relatively high proportion (approximately 35 mol%) of glucose in the hydrolysate could be due to glucomannans (but mannose was not clearly detected in the products), xyloglucan (which is only present at very low levels in wood) or starch (from a coating on the paper). The process by which the pulp was prepared may have swelled the microfibrils and allowed some cellulose hydrolysis by TFA to occur.

Mannose was not detected in the products but lack of separation from xylose in the HPLC chromatogram (Figure 3.4) and the large amount of xylose present may have masked a small amount of mannose.

These results indicate that xylan, some pectic polysaccharides and possibly xyloglucan are present.

3.2.2 Driselase digestion

There was no residue remaining after Driselase digestion of the pulp (Table 3.7). Therefore, the pulp was composed almost entirely of Driselase-susceptible polysaccharides. The digestion products were primarily glucose, xylobiose, xylose, arabinose and glucuronic acid. There were also trace amounts (less than 1 mol% of the digestion products) of rhamnose, galactose, isoprimeverose, cellobiose and galacturonic acid (Table 3.7 and Figure 3.5).

The presence of glucuronic acid in the products was not anticipated as it would be present in the pulp primarily in xylans. There is no known α-glucuronidase activity in Driselase, so glucuronic acid would be expected to be present in di- or trisaccharides of glucuronic acid and xylose (Section 3.1.3.4). The identity of the "glucuronic acid" peak must therefore be regarded as uncertain. A Driselase-only control was analysed by HPLC as Driselase can undergo autolysis upon incubation (Figure 3.5c). The values shown for the pulp screen products (Table 3.7) have had the concentrations of the Driselase-only products subtracted from them. The rhamnose and isoprimeverose in the products may be due to Driselase autolysis as the level of these products in the Driselase-only controls were much higher than the levels calculated to be due to digestion of the pulp.

3.2.3 Discussion

The TFA residue of the pulp was due to the fact that TFA cannot hydrolyse the majority of cellulose because of its crystalline structure. It appears as if a small amount of cellulose had been broken down as there was a small amount (4.3% of the amount in the Driselase digest) of glucose in the hydrolysate. This could be due to the process by which the pulp was prepared, swelling the microfibrils and allowing limited hydrolysis of cellulose by TFA. It has been reported that TFA hydrolysis can break down a small proportion of cellulose from cell walls (5–10%; Selvendran and Ryden, 1990). A small proportion of the glucose in the TFA products may also be derived a starch coating on the paper. The difference between the

glucose levels in the Driselase digest and the TFA hydrolysate was approximately 3345 μmol glucose/ g pulp (≡ 543 mg cellulose/ g pulp). Therefore, at least 54% of the mass of the pulp was cellulose.

Isoprimeverose was only a small proportion (0.04% of the mass of the pulp) of the digestion products; indicating that there was only a trace amount present in the pulp (approximately 0.4 mg xylosyl-glucose back bone/ g pulp). There was no mannose detected in the products of either the TFA or the Driselase screen; however, xylose and mannose are not completely separated on the HPLC, so a large peak of xylose could easily mask a small mannose peak. This would indicate that there was little or no glucomannan in the pulp. The glucose in the Driselase digest was practically all derived from cellulose. The yield of rhamnose (0.6 mg/ g pulp) and galacturonic acid (4.5 mg/ g pulp) in the Driselase digest indicated that there were only trace amounts of pectic polysaccharides present. The xylobiose in the digest indicated that xylan was also present in the pulp. One of the major polysaccharides present in the xylem of the hardwoods is a glucuronoxylan (Timell, 1965) which on Driselase digestion would be expected to yield xylobiose, and di-/ trisaccharides of glucuronsyl-(xylosyl)-xylose (Section 3.1.3.4). The total amount of xylose in the Driselase products was 7.6 × that found in the TFA products. This was surprising as xylan is TFA-susceptible. Xylan in pulp is likely to be deacetylated and therefore able to hydrogen-bond to cellulose microfibrils (Varner and Lin, 1989; Gamerith and Strutzenberger, 1992; Joseleau *et al.*, 1992; Tenkanen and Poutanen, 1992). It is possible that this decreases the susceptibility of xylan to acid hydrolysis.

These results indicate that the polysaccharides present in *Eucalyptus* pulp are primarily cellulose (at least 54% by mass) and xylan. Approximately 25% of the mass of the pulp is due to the xylan backbone. Xyloglucan is present only in minute amounts with only approximately 0.04% of the mass being due to the glucose and xylose from xyloglucan.

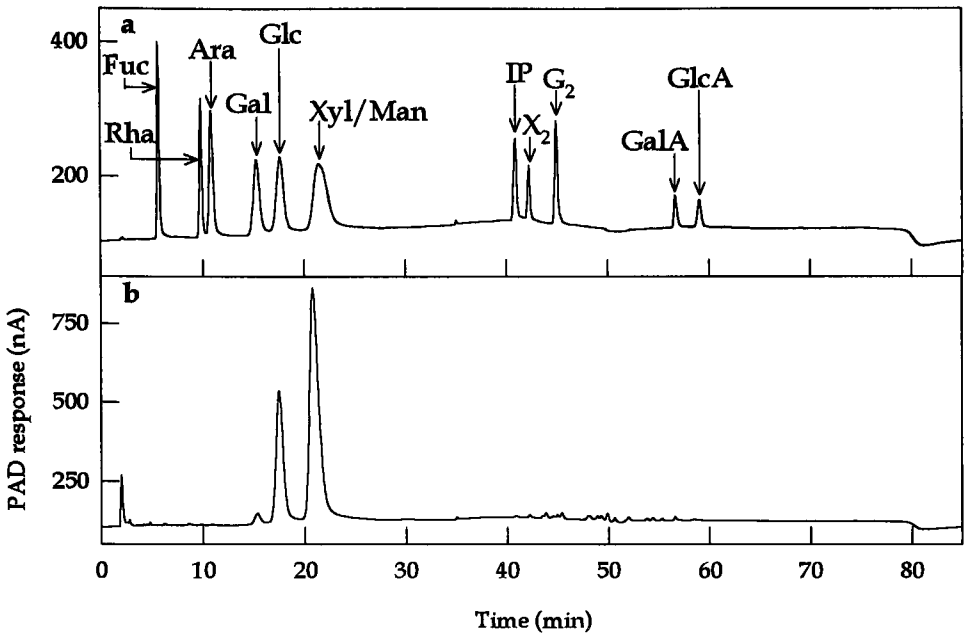

Figure 3.4 TFA hydrolysis products of *Eucalyptus* pulp. The pulp (Section 2.2.3.1) was hydrolysed with 2 M TFA (Section 2.2.4.1). The digestion products were separated on a Dionex HPLC using method 1 (Section 2.2.5.3). **(a)** A mix of standards, each at a concentration of 0.05 mg/ml; **(b)** the TFA hydrolysis products of *Eucalyptus* pulp (a one in two dilution of the original hydrolysate is shown).

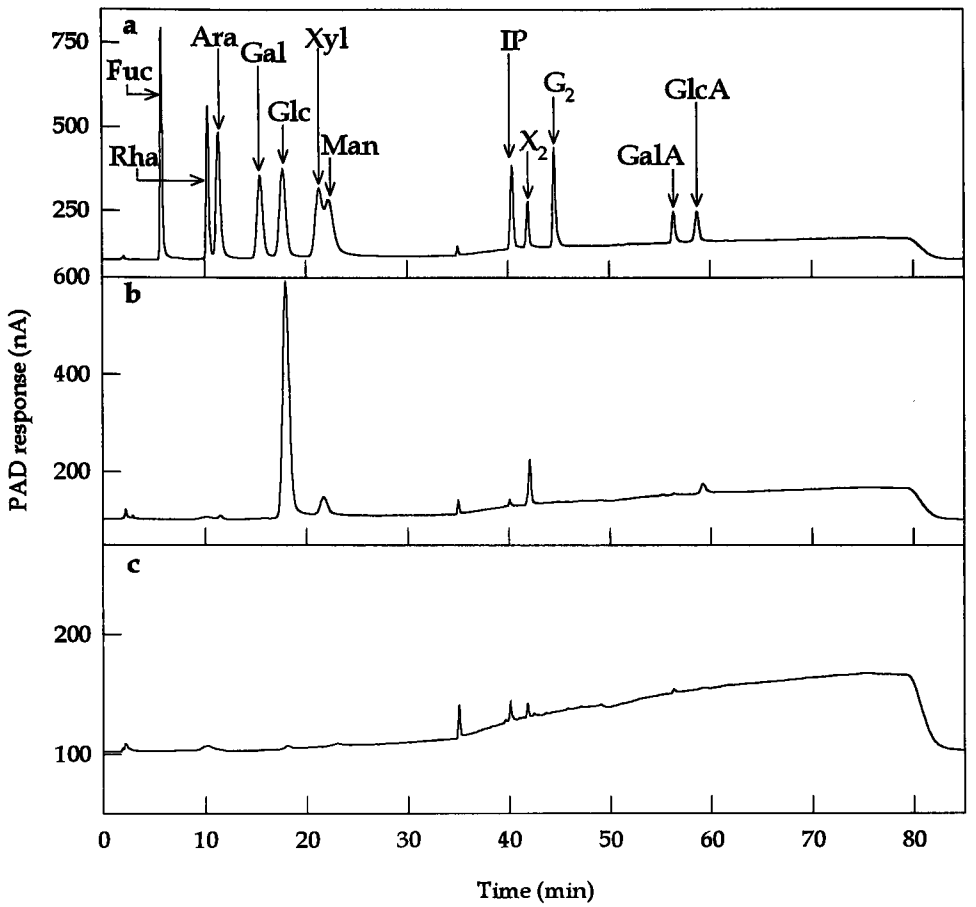

Figure 3.5 Driselase digestion products of *Eucalyptus* pulp. The pulp (Section 2.2.3.1) was incubated with 0.5% Driselase (Section 2.2.4.2). The digestion products were separated on a Dionex HPLC using method 1 (Section 2.2.5.3). **(a)** A mix of standards, each at a concentration of 0.05 mg/ml; **(b)** the Driselase digestion products of *Eucalyptus* pulp (a one in fifty dilution of the original digest is shown); **(c)** a Driselase-only control (one in fifty dilution).

3.3 Discussion

The results of the TFA and Driselase screens for *E. grandis* and *E. globulus* show that there is natural variation in the composition of the xylem cell walls. The two species were grown under the same conditions and wood was collected at the same developmental stage (approximately one year old stems). Therefore any variation between the species will be due to genetic differences.

Xylan is a major component of *Eucalyptus* wood and as such could be a contaminant of cellulose pulps. There was no difference between the two species in the level of total xylose, but there was a lower level of Driselase-resistant xylose in *E. globulus* than in *E. grandis*. This could indicate a greater proportion of acetylated xylose residues and substitution with glucuronic acid in the xylan of *E. grandis*, than in that of *E. globulus*.

The screen products of the pulp indicate that xylan is a major contaminant in *Eucalyptus* pulp, making up approximately 25% of the mass of the pulp. Xylan and other non-cellulosic polysaccharides can form defects in acetate products produced from cellulose pulp and can damage machinery (Wise and Lauer, 1962; Gardner and Chang, 1974; Wong and Saddler, 1992; 1993). Both of these outcomes are costly.

A species of *Eucalyptus* that had wood with a lower level of xylan or had xylan that was more easily extracted (due to a change in structure) would be of benefit to the pulp and paper industry. It would not be so expensive to extract the cellulose pulp and the amount of toxic waste products from the extraction would be decreased.

4 Screening of *A. thaliana* lines

The aim of this section was to screen a mutagenised population of *A. thaliana* for lines that had altered cell wall polysaccharides which are present in paper or have an impact on the extraction and processing of wood in terms of the ease of extractability and/ or use of bleaching agents. Zablackis *et al.* previously found that the polysaccharides present in the cell walls of *A. thaliana* were cellulose, xyloglucan, glucuronoarabinoxylan, homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II.

The populations of *A. thaliana* lines that were screened were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC, Section 2.2.1.2). The substrates for the screen were the AIRs of the tagged *A. thaliana* lines (Section 2.2.3.3). The assays used to screen the *A. thaliana* lines were TFA hydrolysis and Driselase digestion. The screening process consisted of three stages. The aim of the initial screen was to pick out potential cell wall mutants in a quick and labour unintensive way. The purpose of the repeat-screen was to confirm that the differences from the wild type observed in the initial screen were not atypical of the line. In the final stage HPLC was used to analyse quantitatively the screen products of the lines identified as being of interest in the first two stages. Once a small number of lines had been identified for characterisation using HPLC analysis, the initial and repeat-screens were stopped. As a consequence of this not all lines were initially screened by both TFA hydrolysis and Driselase digestion, and not all lines identified as showing an appreciable change in the initial screen were repeat-screened.

The *A. thaliana* lines screened were all genetically heterogeneous (NASC Seed List 1994, for Feldmann lines see Section 2.2.1.2). Therefore when analysing the HPLC data statistically individual plants were compared to the Ws group using Student's t distribution (Section 2.2.6)

4.1 Initial screen

The screen products were separated by TLC (for TFA hydrolysis products; see Sections 2.2.4.1 and 2.2.5.1) and PC (for Driselase digestion products; see Sections 2.2.4.2 and 2.2.5.2). The level of each product on the PC/ TLC was measured semi-quantitatively, with respect to wild type, by visually scoring the intensity of stained spots on the chromatograms (Section 2.3.6). The wild type was scored as zero. Lines were scored as having a more (+1, +2 or +3) or less (-1, -2 or -3) intensely stained spot than the wild type. A score of at least + or - 2 was considered to be a marked change from the wild type. For this screen *A. thaliana* ecotype Columbia (Koncz lines parental; Koncz, 1989) was used as the wild type due to difficulties with germination and growing of ecotype Wassilewskija (Ws, Feldmann lines parental; Feldmann and Marks, 1987; Feldmann *et al.*; 1989) and ecotype Landsberg *erecta* (Bancroft and Dean lines parental; Bancroft and Dean, 1993).

4.1.1 TFA hydrolysis

The AIRs of 107 *A. thaliana* lines (Section 2.2.3.3) were hydrolysed with 2 M TFA (Section 2.2.4.1) and the products separated by TLC (Section 2.2.5.1). Aniline hydrogen-phthalate (Section 2.2.7) was used to stain the TLCs, which were then photographed (Section 2.2.8) under white light and 366-nm UV illumination (Figure 4.1). The less concentrated hydrolysis products (Rha, Fuc and Man) did not give stained spots that were easily visible on the TLC and so were not scored in this screen. The remainder of the products (GalA/ GlcA, Gal, Glc, Ara and Xyl) were scored as described above (Section 4.1). Figure 4.1 shows the hydrolysis products of ten of the *A. thaliana* lines that were screened. Six of those lines (B49, B51, B52, B53, B54, and B64) were scored at least + or - 2 for one or more of the screen products. B51, which scored - 2 for xylose, and B52, which scored - 2 for arabinose and xylose, were repeat-screened (Section 4.2.1).

From the initial TFA screen, 44 lines (41% of lines screened) scored at least + or - 2, with respect to wild type, for one or more hydrolysis products (Table 4.1). A large proportion of the lines identified as differing from wild type (55%; see Table 4.2) showed a difference in the level of xylose (alone or plus one or more other sugars) present in the hydrolysate. A large number of lines also showed differences to the wild type in galactose (27%) and the uronic acids (23%). The majority of the differences observed in the screen products were decreases (100% for glucose; 77% for galactose; 76% for xylose; and 71% for arabinose) except for the uronic acids. Only 20% of the lines that showed an appreciable difference to the wild type in the uronic acids showed decreases. Overall, 70% of the lines identified as showing an appreciable difference to the wild type showed decreases.

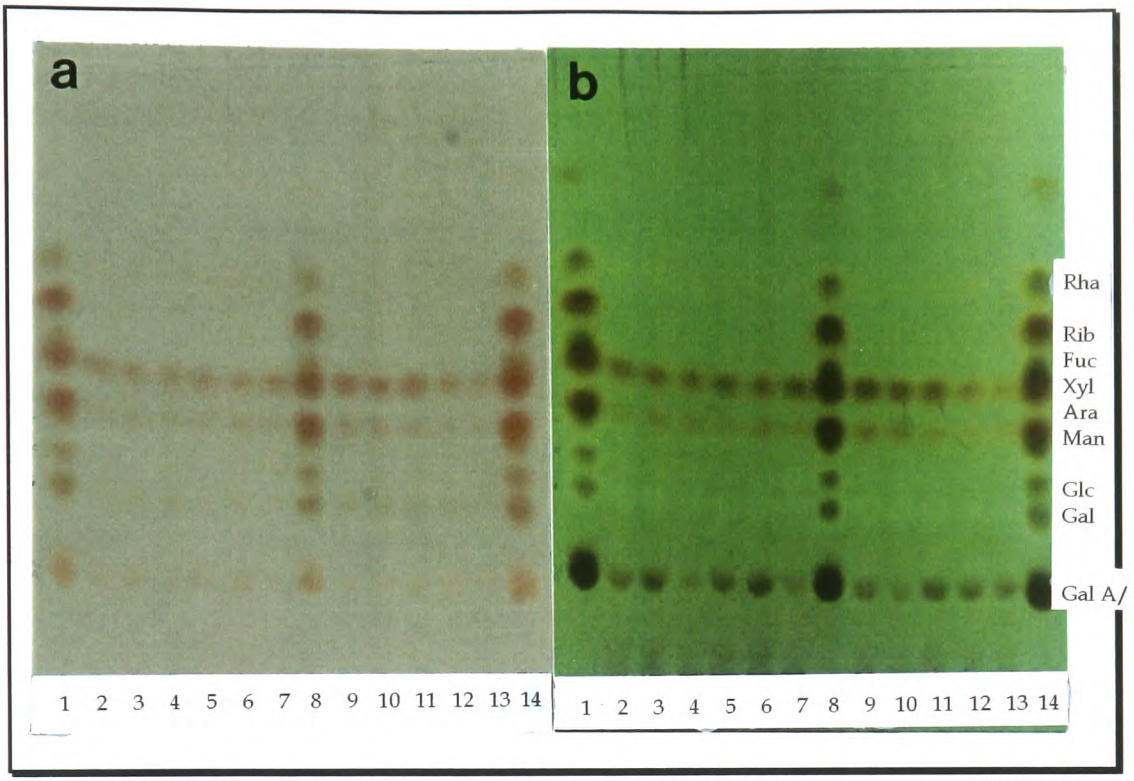

Figure 4.1 Initial screen: TFA hydrolysis products separated by TLC (Sections 2.2.4.1 and 2.2.5.1). The TLC was stained with aniline hydrogen-phthalate (Section 2.2.7) and photographed under **(a)** white light and **(b)** UV illumination (as described in Section 2.2.8). Lanes 1, 8 and 14 = markers (Section 2.2.5.1); lane 2 = B49; lane 3 = B51; lane 4 = B52; lane 5 = B53; lane 6 = B54; lane 7 = B55; lane 10 = B57; lane 11 = B58; lane 12 = B63; lane 13 = B64; lane 9 = wild type (*A. thaliana* ecotype Columbia).

Table 4.1 *A. thaliana* lines tested by the initial screen. AIR was hydrolysed with TFA or incubated with Driselase (Sections 2.3.4.2 and 2.3.4.1). Screen products were separated by TLC or PC (Section 2.3.5). Lines were scored, with respect to *A. thaliana* ecotype Columbia, as described in Section 4.1. [1] = TFA hydrolysis (T) or Driselase digestion (D).

		Products of Driselase digestion or TFA hydrolysis							
Line	Screen[1]	uronic acids	G_2	IP	X_2	Gal	Glc	Ara	Xyl
A1	T	+1				+1	0	+1	+2
A2	D	0	-1	0	0	0	0		
	T	+1				+1	-1	0	0
A3	D	0	-1	0	0	0	0		
	T	-1				0	0	0	0
A4	D	0	0	0	+1	0	0		
A6	D	0	-1	-1	0	0	0		
	T	+1				+1	0	+1	-2
A7	D	0	-1	-1	0	0	-1		
A8	D	0	-1	-1	0	0	0		
A9	T	+1				+1	0	+1	0
A11	D	0	-1	0	-1	-1	-1		
A12	T	0				0	0	0	+2
A18	D	0	0	0	0	+1	0		
A19	D	0	-1	-1	0	0	-1		
A20	D	0	0	0	-1	0	0		
A21	D	0	0	0	-2	-1	0		
	T	+1				+2	0	+1	+1
A22	D	0	-1	0	0	0	0		
A23	D	0	-1	-1	-1	-1	-1		
A25	D	0	-1	0	0	0	0		
A27	D	0	+1	0	0	0	0		
	T	+1				+2	0	+1	0
A30	D	0	+1	0	0	0	0		
	T	0				+1	0	0	0
A31	D	0	0	0	0	+1	0		
A32	D	0	0	0	0	0	-1		
	T	0				0	0	0	0
A33	D	-1	-1	-1	-2	-1	0		
	T	+1				0	0	0	0
A34	D	0	+1	+1	-1	+1	0		
	T	+2				+2	+1	+2	+2

Table 4.1 continued

		Products of Driselase digestion or TFA hydrolysis							
Line	Screen[1]	uronic acids	G_2	IP	X_2	Gal	Glc	Ara	Xyl
A35	D	-1	-1	-1	-3	-1	-1		
A36	D	-1	-1	-1	-3	0	-1		
	T	0				-1	0	0	0
A44	D	0	0	0	0	0	0		
A45	D	0	+2	+1	+1	0	0		
	T	+1				0	0	+1	0
A47	D	0	0	0	0	0	0		
B1	D	0	0	0	-1	-1	0		
	T	+1				+1	0	+1	0
B2	D	-2	-1	-1	-3	-1	-2		
	T	+1				0	0	+1	0
B3	D	-1	0	0	-2	0	-1		
	T	0				-1	0	-1	-1
B4	D	-2	-1	-1	-3	-1	-2		
B5	D	0	-1	0	-2	-2	-1		
	T	0				0	+1	0	-1
B6	D	0	-1	0	-1	-1	-1		
	T	0				0	0	0	-1
B7	D	0	-1	-1	-1	-2	-1		
	T	+1				-1	0	0	0
B8	D	0	-1	-1	-2	-2	-1		
	T	+1				0	0	0	-2
B9	D	-2	-1	-1	-3	-1	-2		
	T	-1				-1	0	0	-1
B10	D	-1	-1	-1	-3	-1	-2		
	T	-1				-2	0	-1	-2
B11	D	0	-1	0	-2	-1	-1		
	T	0				-2	0	0	-1
B12	D	0	+1	+1	-1	+1	0		
	T	-2				0	0	+1	0
B13	D	0	-1	0	-2	-2	-1		
	T	-1				-2	0	-1	-1
B14	D	0	-1	0	-2	-2	-1		
	T	0				-1	0	0	-2
B15	T	+1				0	0	+1	-1
B16	D	0	-1	0	-2	-2	-1		
	T	-1				-2	0	0	-1
B17	D	0	-1	-1	-2	-2	-1		
	T	-2				-2	0	0	-2

Table 4.1 continued

		Products of Driselase digestion or TFA hydrolysis							
Line	Screen[1]	uronic acids	G_2	IP	X_2	Gal	Glc	Ara	Xyl
B18	D	0	-1	0	-2	-1	0		
	T	0				-2	0	-1	-1
B19	D	0	-1	0	-2	-2	-1		
	T	0				-2	0	-1	-1
B20	D	0	0	0	0	+1	0		
B21	D	0	-1	0	-2	-2	0		
	T	0				-1	0	0	0
B22	D	0	0	0	-2	-2	-1		
B23	D	0	-1	0	-1	-1	0		
	T	+1				0	0	+1	0
B24	D	0	-2	0	-2	-1	-1		
	T	-1				-1	+1	0	0
B25	D	0	0	0	-3	-2	-1		
	T	0				-1	-1	0	-1
B26	D	0	0	0	-1	-1	0		
	T	+1				0	+1	+1	0
B27	D	-1	-2	-1	-3	-2	-1		
	T	+1				-1	0	-1	-2
B28	D	-1	-2	-1	-2	-2	-1		
	T	+1				-1	0	-1	-2
B29	D	-1	-2	-1	-2	-2	-1		
	T	0				-1	0	-1	-1
B30	D	-1	-1	0	-2	-1	0		
	T	+1				-2	0	-1	0
B31	D	0	-1	-1	0	-1	0		
B32	D	0	0	0	-1	-1	0		
	T	0				-1	0	0	0
B33	D	-1	-1	0	-2	-1	-1		
B34	D	+1	+1	+2	0	0	0		
	T	+1				0	0	+1	-2
B35	D	0	0	0	-2	-2	-1		
	T	0				-1	0	0	0
B36	D	0	0	0	0	-1	0		
	T	+1				0	+1	+1	-2
B37	D	-1	0	-1	-2	-2	-1		
	T	+1				-1	0	-1	-1
B38	D	0	+1	0	+1	0	0		
	T	0				-1	0	-1	0
B39	D	0	0	0	-2	-1	0		
	T	+1				+1	+2	+1	0

Table 4.1 continued

		Products of Driselase digestion or TFA hydrolysis							
Line	Screen[1]	uronic acids	G_2	IP	X_2	Gal	Glc	Ara	Xyl
B40	D	0	+1	0	-2	0	0		
	T	+1				0	+1	0	-1
B41	D	0	-1	0	-1	-1	0		
	T	+1				0	0	0	0
B42	D	0	-1	-1	-2	-2	-1		
	T	0				0	+1	0	0
B43	D	-1	-1	-1	-2	-1	-1		
	T	+1				-1	0	-1	-1
B44	D	0	+1	0	-2	0	0		
	T	+1				-1	+1	-1	0
B45	D	0	+1	0	0	0	0		
	T	0				0	0	0	-1
B46	D	0	0	0	-2	-1	0		
	T	0				-1	0	-2	0
B47	D	0	+1	+1	-2	-1	0		
	T	0				0	+1	-1	0
B48	D	0	+1	0	-1	0	0		
	T	-1				-1	0	-1	0
B49	D	0	+1	0	+1	0	0		
	T	0				-1	0	-2	-1
B51	D	0	-1	-1	-1	-1	0		
	T	+1				-1	0	-1	-2
B52	D	0	+2	-1	-2	0	0		
	T	0				-1	0	-2	-2
B53	D	0	+1	0	-1	0	0		
	T	0				-1	0	-2	-1
B54	D	-1	-2	-2	-2	-3	-1		
	T	+1				-1	0	-1	-2
B55	D	0	-1	-1	-2	-3	-1		
	T	0				-1	0	-1	-1
B56	D	0	-1	-1	-3	-3	-2		
	T	-1				0	0	0	-1
B57	D	0	-1	-1	-2	-2	-2		
	T	0				0	0	0	0
B58	D	0	0	0	-2	-2	-1		
	T	+1				0	0	0	0
B59	D	0	+1	0	0	0	0		
	T	0				0	0	0	-1

Table 4.1 continued

		Products of Driselase digestion or TFA hydrolysis							
Line	Screen[1]	uronic acids	G_2	IP	X_2	Gal	Glc	Ara	Xyl
B60	D	0	+1	+1	0	0	0		
	T	0				0	0	-1	-1
B61	D	0	+1	+1	**-2**	-1	0		
	T	0				**-2**	0	-1	**-2**
B62	D	0	0	0	+1	0	+1		
	T	+1				0	0	0	0
B63	D	0	+1	+1	-1	-1	0		
	T	0				-1	0	-1	-1
B64	D	0	**+2**	+1	-1	0	0		
	T	0				-1	0	-1	**-2**
B65	D	0	0	0	-1	-1	0		
B69	D	0	0	0	0	-1	0		
B72	D	0	+1	0	0	-1	0		
B73	T	0				0	0	+1	0
B75	T	0				-1	0	0	+1
B76	D	0	+1	0	+1	0	0		
	T	**+2**				0	-1	+1	0
B77	D	0	+1	0	+1	0	0		
B79	D	0	0	0	0	0	0		
C10	T	**+2**				+1	0	+1	**+2**
C13	T	**+2**				+1	0	0	0
C19	T	**+2**				0	-1	0	0
C29	T	+1				0	-1	0	**-2**
C46	T	+1				0	-1	-1	-1
C47	T	0				0	-1	-1	-1
C51	T	-1				-1	0	**-2**	-1
C58	T	0				0	-1	-1	-1
C121	D	0	0	0	+1	0	0		
	T	**+2**				+1	0	0	0
C122	D	0	0	0	0	0	0		
	T	0				0	-1	-1	-1
C123	D	0	0	0	0	-1	0		
	T	+1				0	0	+1	0
C124	D	0	+1	0	0	0	0		
	T	+1				0	0	+1	0
C125	D	0	0	0	0	-1	0		
	T	0				0	-1	0	-1

Table 4.1 continued

		Products of Driselase digestion or TFA hydrolysis							
Line	Screen[1]	uronic acids	G_2	IP	X_2	Gal	Glc	Ara	Xyl
C126	D	+1	+1	0	0	0	0		
	T	+1				0	-2	+1	-1
C128	D	0	+1	-1	0	0	0		
	T	0				0	-1	0	-2
C129	D	0	+1	-1	0	0	0		
	T	0				-1	-2	0	-2
C130	D	0	0	0	+1	0	0		
	T	+2				0	0	0	+2
C131	D	0	+1	0	+1	0	0		
	T	+1				0	0	0	-2
C132	D	0	0	0	+1	0	0		
	T	0				-1	0	0	-1
C133	D	0	+1	0	+1	+1	0		
	T	0				0	0	0	-1
C134	D	0	0	0	0	0	0		
	T	+1				0	0	0	-1
C135	D	0	-1	-1	+1	0	0		
	T	0				0	0	+1	-1
C136	D	0	+1	0	+1	+1	0		
	T	0				0	-1	0	0
C137	D	0	+1	0	0	+1	0		
	T	0				-1	-1	0	+1
C138	T	+2				0	0	+2	-1
C139	D	0	0	0	0	-1	0		
	T	+1				0	0	+1	-2
C140	T	+1				-1	0	-1	0
C142	T	0				-1	-1	-1	0
C143	T	+1				0	0	+1	0
C145	T	0				-1	0	0	0

Table 4.2 Initial TFA screen: Number of lines to show appreciably[1] altered levels of TFA hydrolysis products relative to wild type (*A. thaliana* ecotype Columbia). [1] = scored at least + or – 2 in the initial screen

Hydrolysis products	No. of lines with altered level of screen products	% of lines with altered level of screen products
uronic acids	5	11.4
galactose	8	18.2
glucose	2	4.5
arabinose	4	9.1
xylose	16	36.4
uronic acids, arabinose	1	2.3
uronic acids, xylose	2	4.5
galactose, xylose	2	4.5
glucose, xylose	1	2.3
arabinose, xylose	1	2.3
uronic acids, galactose, xylose	1	2.3
uronic acids, galactose, xylose, arabinose	1	2.3
Total =	**44**	

4.1.2 Driselase digestion

The AIRs of 110 *A. thaliana* lines (Section 2.3.3.3) were digested with purified Driselase (Section 2.1.1 and Section 2.2.4.2), and the products separated by PC (Section 2.2.5.2). Aniline hydrogen-phthalate (Section 2.2.7) was used to stain the PCs. The PCs were photographed (Section 2.2.8) under white light (Figure 4.2) and 366-nm UV illumination (Figure 4.3). The disaccharide digestion products were easier to score when viewed under UV illumination. The aim of the Driselase digestion screen was to examine the relative levels of the disaccharides produced which are diagnostic of the polysaccharides cellulose, xyloglucan and xylan. In order to get a good separation of these disaccharides the PCs were developed for 70 h. After this time some of the digestion products (Fuc, Rha, Ara and Xyl) had usually run off the end of the PC and therefore could not be scored. Mannose, one of the less concentrated digestion products, did not give stained spots that were easily visible on the PC and so was not scored in this screen. The remainder of the products (GalA/ GlcA, G_2, IP, X_2, Gal and Glc) were scored as described in Section 4.1. Figures 4.2 and 4.3 show the digestion products of 11 of the *A. thaliana* lines that were screened. Seven of those lines (B52, B55, B56, B57, B58, B61 and B64) were scored at least + or - 2 for one or more of the screen products. B58 was identified as showing a decrease in xylobiose and galactose (Figure 4.3 and Table 4.3). B52 was identified as showing an increase in cellobiose and a decrease in xylobiose (Figure 4.3 and Table 4.3). Both B52 and B58 were repeat-screened (Section 4.2.2).

Of the 110 lines screened in this way 47 (43%) scored + or - 2, with respect to wild type for one or more digestion products (Table 4.1). This was considered to be a marked difference from the wild type. The majority of the lines identified as differing from the wild type showed a difference in the level of xylobiose (91%; Table 4.3) or galactose (45%). The majority of the differences observed (92%) were decreases from the level observed in the wild type. The only screen products for which increased levels were

observed were cellobiose (43% of the lines identified as showing a difference in cellobiose) and isoprimeverose (50% of the lines identified as showing a difference in isoprimeverose).

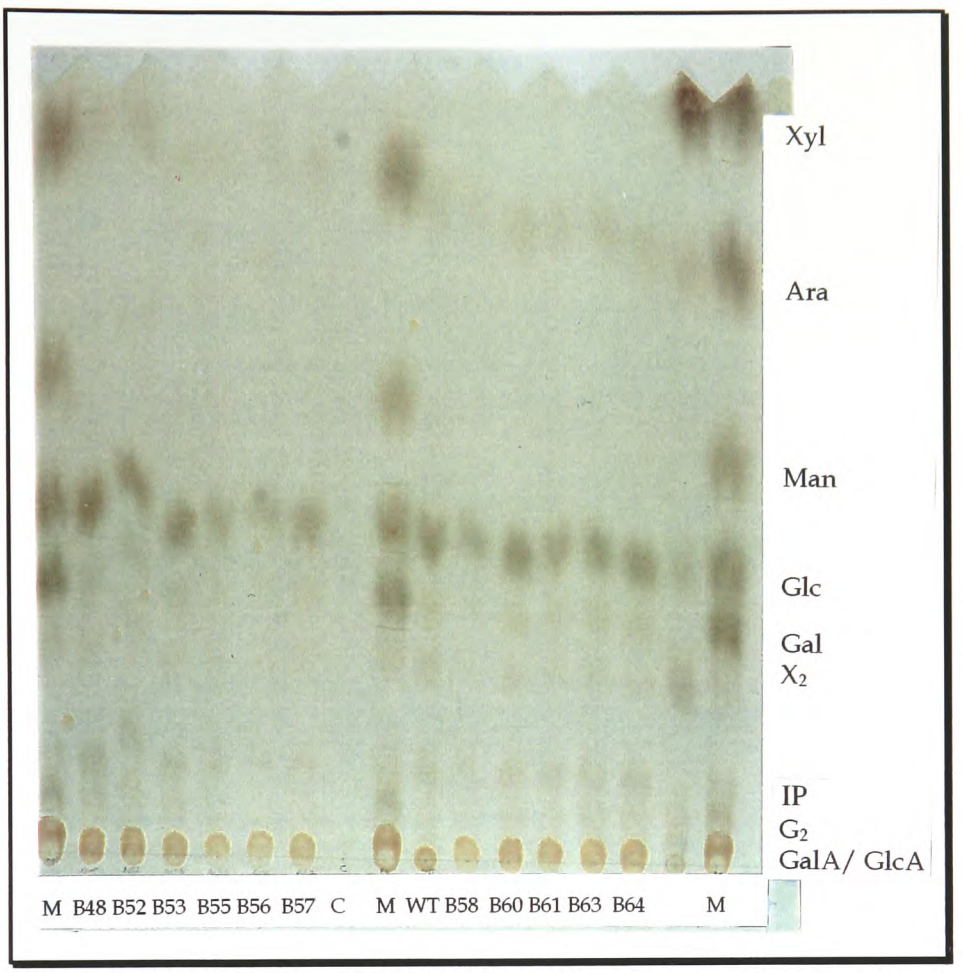

Figure 4.2 Initial screen: Driselase digestion products separated by PC (Sections 2.2.4.2 and 2.2.5.2). The PC was stained with aniline hydrogen-phthalate (Section 2.2.7) and photographed under white light as described in Section 2.2.8. M = markers (Section 2.3.5.2); C = Driselase-only control (Driselase undergoes some autolysis upon incubation); WT = *A. thaliana* ecotype Columbia; B48 - B64 = screened *A. thaliana* lines.

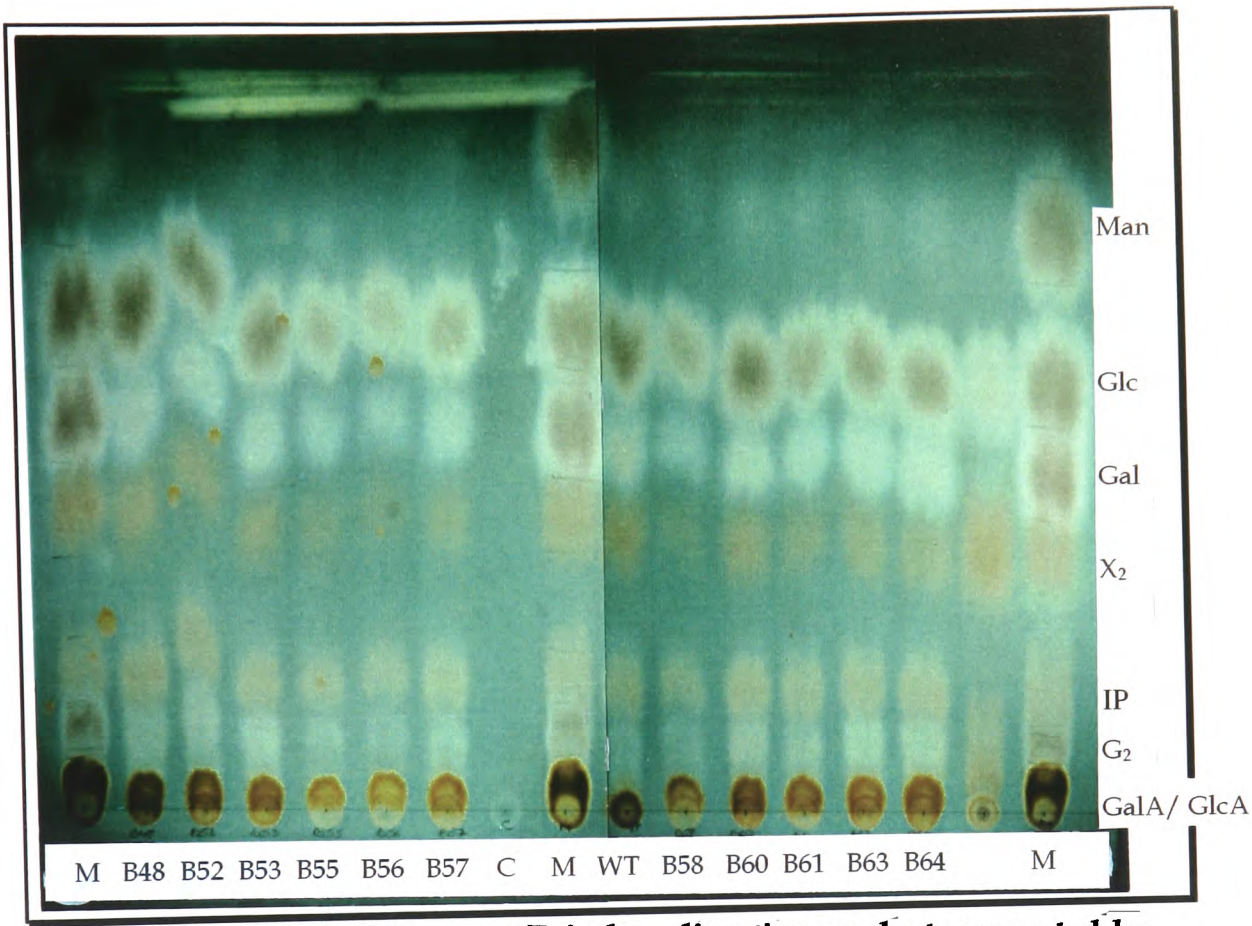

Figure 4.3 Initial screen: Driselase digestion products separated by PC as described for Figure 4.2, except the PC was photographed under UV illumination as described in Section 2.2.8.

Table 4.3 Initial Driselase screen: Number of lines to show appreciably[1] altered levels of Driselase digestion products, relative to *A. thaliana* ecotype Columbia. [1] = scored at least + or – 2 in the initial screen.

Digestion products	No. of lines with altered level of screen products	% of lines with altered level of screen products
cellobiose	2	4.3
isoprimeverose	1	2.1
xylobiose	17	36.2
galactose	1	2.1
cellobiose, xylobiose	2	4.3
xylobiose, galactose	15	31.9
xylobiose, glucose	1	2.1
uronic acids, xylobiose, glucose	2	4.3
cellobiose, xylobiose, galactose	3	6.4
xylobiose, galactose, glucose	2	4.3
cellobiose, isoprimeverose, xylobiose, galactose	1	2.1
Total =	**47**	

4.2 Repeat-screen

4.2.1 TFA hydrolysis

Plants from nine of the 44 lines identified as showing differences from the wild type in the initial screen were repeat-screened. The AIRs were extracted and pooled for each line, and 2–3 replicates were hydrolysed. The hydrolysis products of the nine repeat-screened lines were separated by TLC (Section 2.2.5.1). The TLCs were stained with aniline hydrogen-phthalate (Section 2.2.7) and photographed (Section 2.2.8). The TLCs were scored as described for the initial screen (Section 4.1).

Of the nine lines repeat-screened, five showed a consistent difference, when compared to the wild type (*A. thaliana* ecotype Columbia), in the level of one or more hydrolysis products (Table 4.4). Of these five, three lines (B14, see Figure 4.4; B51; and B52) showed a decrease in the concentration of xylose in the hydrolysate. This result was consistent with the initial screen. B52 was also identified as having a decreased level of xylobiose and isoprimeverose by the repeat Driselase screen (Sections 4.2.2). The other two lines (A21 and B16) showed changes in the concentration of arabinose and the uronic acids. This result was not consistent with the initial screen. A21 and B51 were not analysed further owing to time constraints. The screen products of B14, B16 and B52 were analysed by HPLC (Section 4.3.2).

4.2.2 Driselase digestion

Of the 41 lines identified as showing a difference from the wild type by the initial screen, 18 were repeat-screened. The AIRs were extracted and pooled for each line, and 2 - 3 replicates were digested. The digestion products were separated by PC (Section 2.2.5.2). Aniline hydrogen-phthalate (Section 2.2.7) was used to stain the PCs, which were then photographed (Section 2.2.8) under white light and 366–nm UV illumination (Figure 4.5). The PCs were scored as described for the initial screen (Section 4.1).

Nine lines were identified as showing a consistent difference from the wild type (Table 4.4). The majority (8 out of 9, 89%) showed an alteration in one or two of the disaccharide screen products. The concentration of xylobiose was decreased in seven of the nine lines. B17, B34 and B52 showed changes in both xylobiose and isoprimeverose. This result was not absolutely consistent with the initial screen, where B17 was seen to have decreased xylobiose and decreased galactose. B34 was originally identified as showing only increased isoprimeverose and B52 was initially observed to have decreased xylobiose and increased cellobiose. B52 was also identified by the repeat TFA hydrolysis screen, where it showed a decrease in the level of xylose (Sections 4.2.1). B5 had a decreased level of isoprimeverose only. This result was not consistent with the initial screen where it was identified as showing decreased levels of xylobiose and galactose. B24 (Figure 4.5) and B28 had decreased levels of cellobiose and xylobiose, which was consistent with the initial screen, although B28 was also seen to have decreased galactose in the initial screen. Alterations were also seen in galactose in B27, B54 and B58. Again, these results were not absolutely consistent with initial screen. Of the nine repeat-screened lines that had shown a decrease in xylobiose in the initial screen, only one (B27) showed a consistent difference from the wild type in the repeat-screen. B27 was also identified as showing a difference from the wild type by the initial TFA hydrolysis screen (decreased xylose, Section 4.1.1) but showed no consistent difference in the repeat TFA screen. B58 showed a consistent decrease in the level of galactose. It was originally identified in the initial screen as showing a difference in galactose and xylobiose. Of the nine lines identified seven (B5, B17, B24, B27, B34, B52 and B58) were analysed using HPLC (Section 4.3.3). The other lines were not analysed further owing to time constraints.

Table 4.4 *A. thaliana* lines identified as showing a difference from the wild type by the repeat-screen. AIR was hydrolysed with TFA (Section 2.2.4.1) or incubated with Driselase (Section 2.2.4.2). TFA hydrolysis products were separated by TLC and Driselase digestion products were separated by PC (Section 2.2.5). Only consistent differences in the level of a screen product are shown, i.e. the initial and all repeat-screens showed the increase or decrease in the screen product.

Line	Screening method	Result of repeat-screen
A21	TFA hydrolysis	**decrease in GalA/ GlcA and Ara**
A33	Driselase digestion	no consistent differences from the wild type
B5	Driselase digestion	**decrease in IP**
B11	Driselase digestion	no consistent differences from the wild type
B12	TFA hydrolysis	no consistent differences from the wild type
B14	TFA hydrolysis	**decrease in Xyl**
B16	TFA hydrolysis	**increase in GalA/ GlcA and Ara**
B17	Driselase digestion	**decrease in IP and X_2**
B19	TFA hydrolysis	no consistent differences from the wild type
B24	Driselase digestion	**decrease in G_2 and X_2**
B25	Driselase digestion	no consistent differences from the wild type
B27	Driselase digestion	**decrease in Gal and X_2**
	TFA hydrolysis	no consistent differences from the wild type
B28	Driselase digestion	**decrease in G_2 and X_2**
B29	Driselase digestion	no consistent differences from the wild type
B34	Driselase digestion	**increase in IP, decrease in X_2**
B39	TFA hydrolysis	no consistent differences from the wild type
B40	Driselase digestion	no consistent differences from the wild type
B44	Driselase digestion	no consistent differences from the wild type
B47	Driselase digestion	no consistent differences from the wild type
B51	TFA hydrolysis	**decrease in Xyl**
B52	Driselase digestion	**decrease in IP and X_2**
	TFA hydrolysis	**decrease in Xyl**
B54	Driselase digestion	**decrease in X_2 and Gal**
B55	Driselase digestion	no consistent differences from the wild type
B58	Driselase digestion	**decrease in Gal**
B64	Driselase digestion	no consistent differences from the wild type

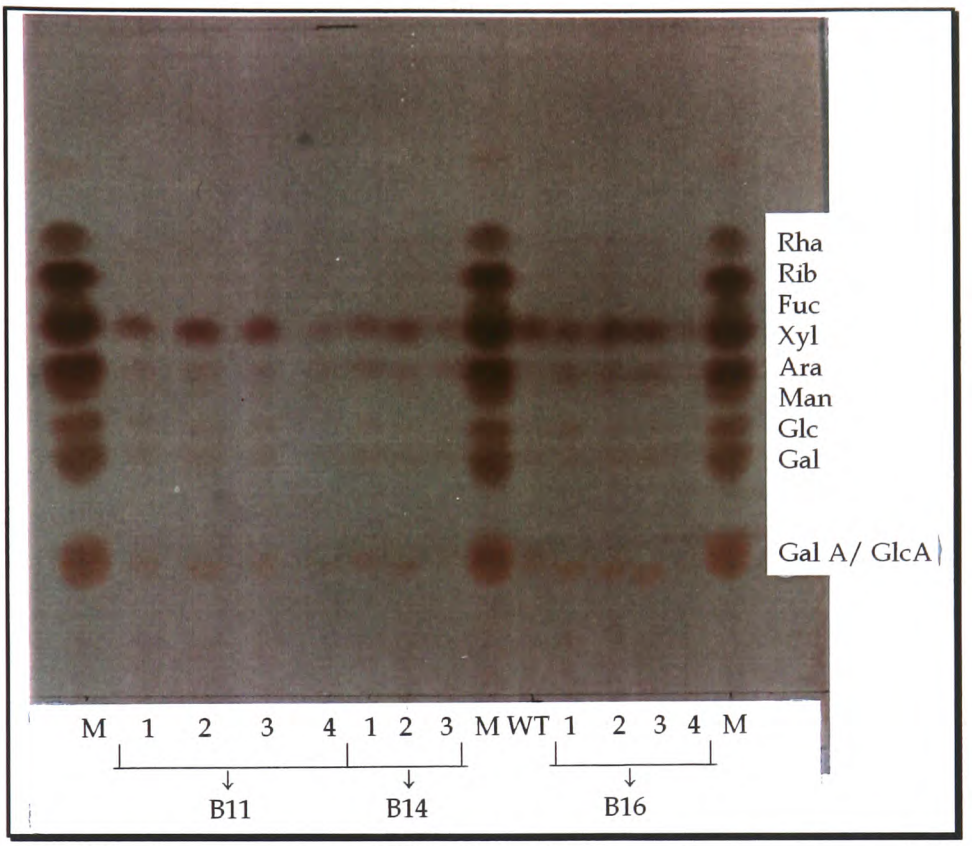

Figure 4.4 Repeat-screen: TFA hydrolysis products separated by TLC (Sections 2.2.4.1 and 2.2.5.1). The TLC was stained with aniline hydrogen-phthalate (Section 2.2.7) and photographed under white light as described in Section 2.2.8. M = markers (Section 2.2.5.1); WT = *A. thaliana* ecotype Columbia; B11 (1 - 3), B14 (1 - 2) and B16 (1 -3) = repeat-screen hydrolysates; B11 (4), B14 (3) and B16 (4) = initial screen hydrolysate.

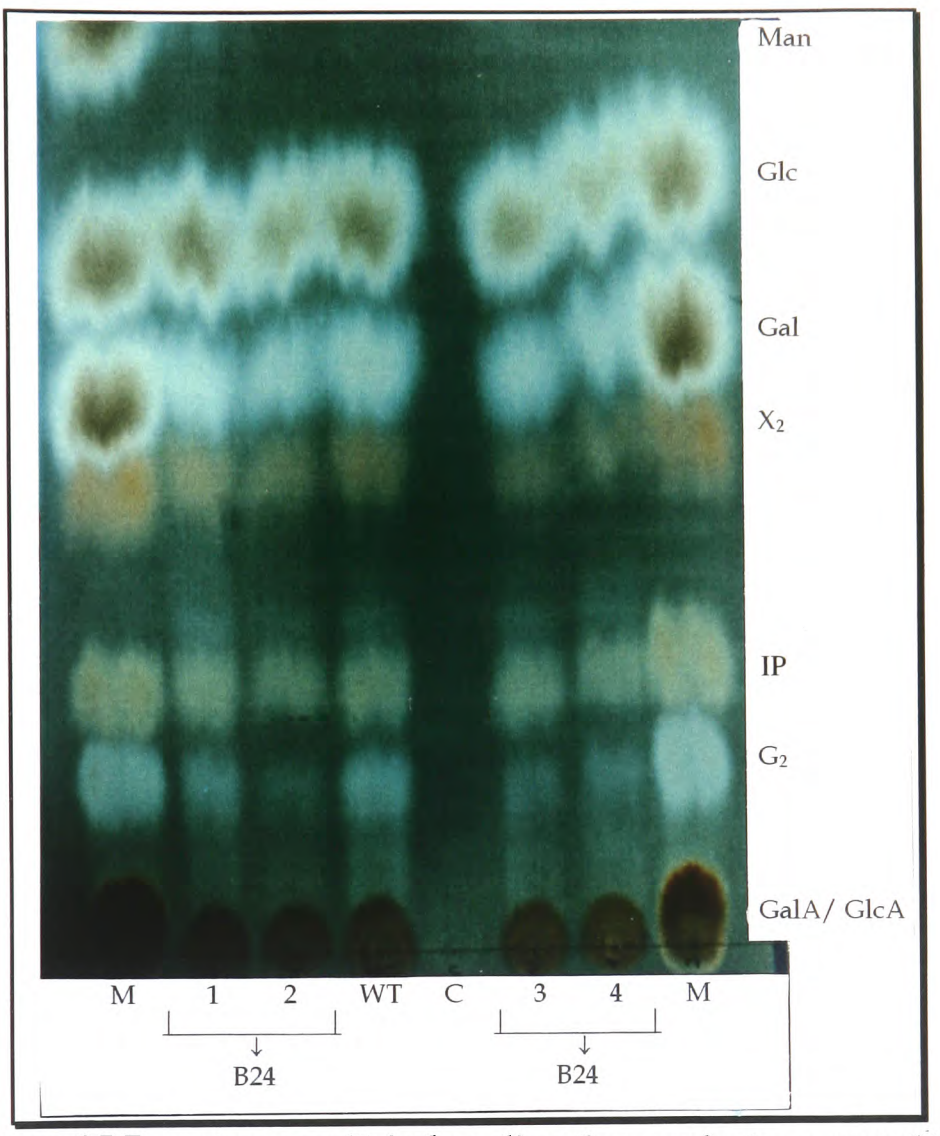

Figure 4.5 Repeat-screen: Driselase digestion products separated by PC (Sections 2.2.4.2 and 2.2.5.2). The PC was stained with aniline hydrogen-phthalate (Section 2.2.7) and photographed under UV illumination as described in Section 2.2.8. M = markers (Section 2.2.5.2); C = Driselase-only control (Driselase undergoes some autolysis upon incubation); WT = *A. thaliana* ecotype Columbia; B24 (1 - 3) = repeat-screen hydrolysates; B24 (4) = initial screen hydrolysate.

4.3 HPLC analysis

4.3.1 General

Of the *A. thaliana* lines identified as showing a consistent difference from Columbia in the repeat-screen, three lines identified by TFA hydrolysis (B14, B16 and B52) and seven identified by Driselase digestion (B5, B17, B24, B27, B34, B52 and B58) were analysed by HPLC. A21 and B51 (identified by the TFA hydrolysis screen), and B28 and B54 (identified by the Driselase digestion screen) were not analysed further owing to time constraints. The nine lines that were to be analysed using HPLC were all screened by both Driselase digestion and TFA hydrolysis from this point so that comparisons between the results for each line would, in some cases, indicate which polysaccharide had been altered. The nine lines to be analysed by HPLC were all Feldmann T-DNA tagged lines (Feldmann and Marks, 1987; Feldmann *et al.*, 1989).

Plants from each of the nine lines were grown on soil (Section 2.2.2.2). The AIR was prepared from the flowering stems of individual plants (Section 2.2.3.3) and hydrolysed with TFA (Section 2.2.4.1) or Driselase (Section 2.2.4.2).

4.3.2 TFA hydrolysis

TFA hydrolysis products were separated on a Dionex HPLC (Section 2.2.5.3) using method 1 (Figure 2.1). Figure 4.6 shows the TFA hydrolysis products of the AIR from *A. thaliana* ecotype Wassilewskija tissue (Ws, the Feldmann parental line).

The proportion of TFA hydrolysis products, the total mass of products and the mass of the hydrolysis residues for each of the nine lines, plus Ws and Columbia, are shown in Table 4.5. Table 4.5 gives the proportions (mol%) and the yields (μmol/ g AIR) of the screen products. The use of proportions removes variability due to differences in the proportion of the AIR solubilised and allows comparisons to be made between *A. thaliana*

lines. The yields of the products allow comparisons between the results of the two screens for a particular *A. thaliana* line.

4.3.2.1 Hydrolysis residue

The residue remaining after TFA hydrolysis of the AIR was washed, dried under a vacuum and weighed. A difference between Ws and one of the other lines in the mass of the residue would imply that there was a difference in the proportion of TFA-susceptible polymers in the AIR.

The hydrolysis residues of Ws and Columbia did not have significantly different masses. The Columbia residues were, on average, 9% heavier than the Ws residues (Table 4.5). The residue mass was significantly different from the parental line, Ws, in individuals from six of the Feldmann lines (B5, B14, B24, B27, B34 and B58). For five of these lines (B5, B14, B27, B34 and B58) only one or two individuals had a significantly different residue mass from Ws. However, four B24 individuals had a significantly different residue mass from Ws, although three had smaller residues and one larger. The residues of the Feldmann lines ranged from 39% (B24-1) to 159% (B14-1) of the mass of the residue of Ws (Table 4.5).

4.3.2.2 Total mass of identified carbohydrate hydrolysis products

The total mass of identified carbohydrate products obtained for each line is a measure of the TFA-susceptible polysaccharides present in the AIR. A difference from Ws in the total carbohydrate products would indicate that the accessible TFA-susceptible polysaccharides made up a different proportion of the AIR than in the parental line.

There was no significant difference between the total mass of identified products for Ws and Columbia (Table 4.5). The total mass of identified products was significantly different from Ws in individuals from seven of the Feldmann lines (B5, B14, B16, B27, B34, B52 and B58). For five of these lines (B5, B14, B27, B34 and B52) only one or two individuals had a significant difference from Ws. The other two lines (B16 and B58) had four

(all increases) and three individuals (all decreases), respectively, with a significant difference from Ws. The total mass of identified carbohydrate hydrolysis products for the Feldmann lines ranged from 31% (B27 b1) to 178% (B16 d5) of that for Ws.

4.3.2.3 Identified carbohydrate hydrolysis products

***A. thaliana* ecotype Ws**

The major components of the TFA hydrolysate of Ws were xylose, galacturonic acid, glucose and galactose. Together they made up approximately 78 mol% of the total identified carbohydrate components of the hydrolysate (Table 4.5). The xylose in the hydrolysate is derived from xylan, xyloglucan and the rhamnogalacturonans. The second most concentrated component was galacturonic acid, which is a breakdown product of the pectic polysaccharides (homogalacturonan and rhamnogalacturonan I and II). Galacturonic acid glycosyl bonds are relatively resistant to the acid hydrolysis conditions used so the yield of galacturonic acid in the hydrolysate was unlikely to reflect its true level in the AIR.

Glucose, which only made up 11 mol% of the hydrolysate, is derived from xyloglucan, starch and a minor amount from rhamnogalacturonan II. Cellulose is not hydrolysed by TFA because of its crystalline structure. The galactose and the arabinose in the hydrolysate are derived from xyloglucan (galactose only; Zablackis *et al.*, 1996), xylan (arabinose only), the rhamnogalacturonans and the arabinogalactans. The arabinogalactans are believed to bind to the rhamnogalacturonans via rhamnose residues. Rhamnose is a component of the pectic polysaccharides. Mannose can be found in the cell wall in mannans and the oligosaccharide chains of glycoproteins.

Fucose and glucuronic acid were minor components of the TFA hydrolysate, being only 0.8 and 1.4 mol% of the products, respectively. Fucose is a component of the side chains of xyloglucan and the

rhamnogalacturonans, while glucuronic acid is present in xylans and also the rhamnogalacturonans.

A. thaliana ecotype Columbia

The TFA hydrolysate of the *A. thaliana* ecotype Columbia had a small number of significant differences from that of Ws (Table 4.5). The proportions of galactose and glucose were 10% and 42% higher, respectively, in Columbia than in Ws, while the proportion of mannose was 21% lower in Columbia than in Ws.

The increased proportion of glucose in the hydrolysate of Columbia implied that one or more of the TFA-susceptible glucose-containing polysaccharides were increased by a significant amount. The decrease in mannose could be due to a difference from Ws in mannans or glycoproteins.

T-DNA tagged lines

All of the nine Feldmann lines screened by HPLC showed significant differences from Ws in the composition of the TFA hydrolysate for at least 50% of the individuals analysed (Table 4.5).

The proportion of glucose in the hydrolysates of the Feldmann lines ranged from 42% (B16-5) to 141% (B14-5) of that for Ws. The proportion of glucose was significantly different from that of Ws in individuals from six of the Feldmann lines (B5, B14, B16, B17, B34 and B58). However, only one (B5-1, B14-5, B16-5, B17-5, and B58-7) or two (B34 a4 and B34 c5) individuals from each line showed the significant difference from Ws. All of the significant differences from Ws were decreases in the proportion of glucose, except for B14-5 and B34 a4, which were 141% and 140% of Ws respectively.

All but one of the Feldmann lines had at least one individual that showed a significant difference from Ws in the proportion of xylose in the hydrolysis products. For three of the lines (B5, B16 and B17) only one individual showed a significant difference from Ws, while two (B27), three (B24, B34 and B58) and five (B52) individuals from the other lines showed

significant differences. Of all the significant differences only one (B34 c5) was an increase compared to Ws. The proportion of xylose in the hydrolysates of the Feldmann lines ranged from 32% (B58 d3) to 127% (B34 c5) of that for Ws. The decreases in the proportion of xylose could be due to lower proportions of xylan or xyloglucan.

All of the nine Feldmann lines had two or more individuals that showed significant differences from Ws in the proportion of galacturonic acid present. The proportions of galacturonic acid ranged from 60% (B24 a4) to 184% (B34-3) of that for Ws. All the individuals that showed significant differences from Ws in the proportion of rhamnose had decreased levels compared to Ws. The proportions of rhamnose in the hydrolysates ranged from 63% (B34 c5) to 119% (B24-1) of that for Ws. There did not appear to be any correlation between the proportion of the hydrolysate that was galacturonic acid and the proportion that was rhamnose.

Individuals from eight of the Feldmann lines (B5, B14, B16, B17, B24, B27, B34 and B58) showed significant increases in arabinose and galactose compared to Ws. For these lines all of the individuals that had significant differences in the proportion of galactose also showed a significant difference in the proportion of arabinose. Six out of seven of the B52 individuals had significantly increased galactose but no significant difference in arabinose. The seventh B58 individual had significantly lower arabinose but no difference from Ws in galactose. For arabinose the proportions present in the Feldmann lines ranged from 64% (B52-5) to 264% (B58 d3) of that for Ws, while for galactose the proportions ranged from 85% (B16 a6) to 255% (B24-1).

The proportion of mannose in the hydrolysate was significantly different from that of Ws in only six individuals (B5-3, B5 b4, B14-6, B16 b6, B16 d5 and B58 b2). All of these significant differences were lower than Ws. The proportion of mannose in the hydrolysates varied from 45% (B16 d5) to 132% (B24 a4) of that for Ws.

Individuals from each of the nine Feldmann lines showed significant differences from Ws in the proportion of fucose and/ or glucuronic acid in the hydrolysate. However, both fucose and glucuronic acid were minor constituents of the hydrolysates. The proportions of these products ranged from 0.2 to 1.8 mol% for glucuronic acid and from 0.6 % to 1.4 mol% for fucose (Table 4.5).

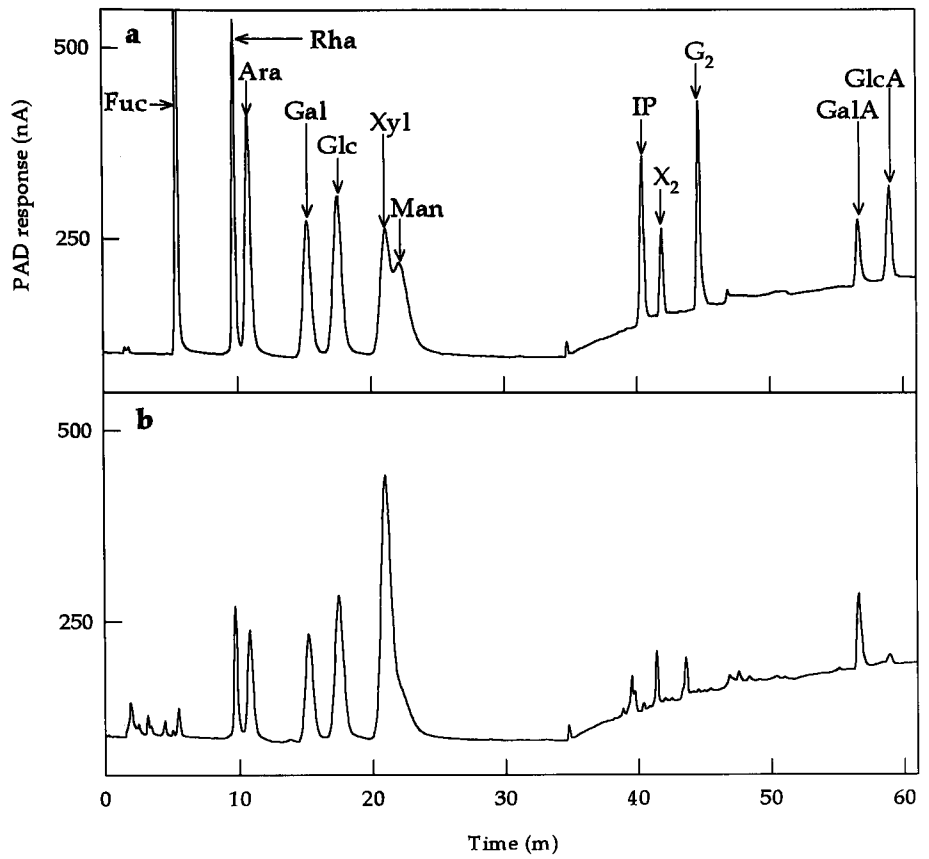

Figure 4.6 TFA hydrolysis products of the AIR of the flowering stem of *A. thaliana* ecotype Wassilewskija. The AIR was hydrolysed with 2 M TFA (Section 2.2.4.1). The hydrolysis products were separated on a Dionex HPLC using method 1 (Section 2.2.5.3). **(a)** A mix of standards, each at a concentration of 0.05 mg/ ml; **(b)** the TFA hydrolysis products of the AIR of the flowering stem of *A. thaliana* ecotype Wassilewskija (a 1 in 5 dilution of the original hydrolysate is shown).

Table 4.5 The TFA hydrolysis products of the AIR of *A. thaliana* lines. The AIR was hydrolysed with 2 M TFA (Section 2.2.4.1). Hydrolysis products were separated on a Dionex HPLC (Section 2.2.5.3). Chromatograms were calibrated using external standards and Dionex software. The figures shown for Ws and Columbia are means, with standard deviations in brackets. n = number of replicates; ** probability of less than 0.05 that the line is from the same population as the parental line, Ws; * probability of $0.05 < p < 0.10$ that the line is from the same population as the parental line, Ws (as calculated by Student's t distribution, Section 2.2.6).

	Products of TFA hydrolysis (mol%; µmol/ g AIR)									Total mass of identified products (mg/ g AIR)	Mass of hydrolysis residue (mg/ g AIR)
Line	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA		
Ws	0.8 (0.1)	6.7 (0.7)	7.2 (0.8)	10.6 (1.2)	11.3 (2.0)	40.4 (4.2)	6.2 (0.8)	15.4 (1.2)	1.4 (0.3)	182 (28.2)	348 (52.0)
n = 6	8.9 (1.1)	72.6 (8.6)	77.8 (11.5)	114 (14.4)	121 (19.7)	444 (103)	68.3 (17.2)	167 (23.8)	15.5 (4.9)		
Col	0.8 (0.2)	5.0 (0.3)	6.7 (0.8)	11.7* (1.1)	16.0** (3.4)	39.3 (3.5)	4.9** (0.4)	14.5 (1.5)	1.0 (0.2)	173 (13.8)	380 (13.8)
n = 6	8.1 (1.5)	51.3 (5.3)	70.0 (11.1)	122 (12.6)	166 (39.6)	412 (58.0)	51.5 (6.0)	150 (12.8)	10.6 (2.3)		
B5-1	0.8	4.6*	7.9	10.9	6.1*	43.8	6.0	19.0**	0.9	318**	400
	15.2	88.3	150	208	117	836	114	363	18.0		
B5-2	0.7	4.6*	6.5	9.6	9.6	45.5	7.4	15.3	0.7**	230	398
	9.1	63.9	89.9	133	133	630	103	211	10.3		
B5-3	1.3**	6.6	17.0**	21.8**	9.1	17.6**	4.3*	22.1**	0.2**	199	234*
	15.2	76.1	197	252	105	203	49.9	255	2.6		
B5 a4	1.0	5.5	9.7**	12.7	7.9	37.6	5.9	18.3*	1.4	213	332
	12.2	70.0	123	161	99.9	476	74.9	232	18.0		
B5 a6	0.8	5.5	8.1	10.8	10.8	42.0	7.0	13.9	1.2	185	368
	9.1	60.9	89.9	119	119	466	77.7	155	12.9		
B5 b4	0.7	4.7*	8.3	11.4	8.0	44.9	4.5*	17.0	0.6**	214	383
	9.1	60.9	107	147	103	580	58.2	219	7.7		
B5 c4	0.8	4.9*	6.8	9.2	10.7	46.9	6.4	12.8	1.5	194	433
	9.1	57.9	79.9	108	125	550	74.9	149	18.0		
B5 Mean	0.9 (0.2)	5.2 (0.7)	9.2 (3.6)	12.4 (4.3)	8.9 (1.7)	39.8 (10.2)	5.9 (1.2)	16.9 (3.2)	1.0 (0.5)	222 (45.1)	364 (65.2)
n = 7	11.3 (2.9)	68.3 (10.8)	119 (41.5)	161 (51.8)	115 (12.4)	534 (192)	78.9 (22.6)	226 (71.8)	12.5 (6.0)		

Table 4.5 continued.

Line	Products of TFA hydrolysis (mol%; µmol/ g AIR)									Total mass of identified products (mg/ g AIR)	Mass of hydrolysis residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA		
Ws	**0.8 (0.1)**	**6.7 (0.7)**	**7.2 (0.8)**	**10.6 (1.2)**	**11.3 (2.0)**	**40.4 (4.2)**	**6.2 (0.8)**	**15.4 (1.2)**	**1.4 (0.3)**	182 (28.2)	348 (52.0)
n = 6	8.9 (1.1)	72.6 (8.6)	77.8 (11.5)	114 (14.4)	121 (19.7)	444 (103)	68.3 (17.2)	167 (23.8)	15.5 (4.9)		
Col	**0.8 (0.2)**	**5.0 (0.3)**	**6.7 (0.8)**	**11.7* (1.1)**	**16.0** (3.4)**	**39.3 (3.5)**	**4.9** (0.4)**	**14.5 (1.5)**	**1.0 (0.2)**	173 (13.8)	380 (13.8)
n = 6	8.1 (1.5)	51.3 (5.3)	70.0 (11.1)	122 (12.6)	166 (39.6)	412 (58.0)	51.5 (6.0)	150 (12.8)	10.6 (2.3)		
B14-1	**0.7**	**5.0***	**6.7**	**10.7**	**13.4**	**43.3**	**6.6**	**12.4***	**1.3**	224	554**
	9.1	67.0	89.9	144	180	583	88.8	167	18.0		
B14-2	**1.0**	**5.6**	**8.8**	**12.5**	**10.7**	**39.8**	**6.5**	**14.5**	**0.8***	208	350
	12.2	70.0	110	155	133	496	80.5	180	10.3		
B14-5	**0.7**	**5.0***	**6.2**	**9.7**	**15.9***	**41.7**	**6.2**	**13.4**	**1.2**	224	409
	9.1	67.0	83.3	130	214	560	83.2	180	15.5		
B14-6	**0.9**	**5.2**	**11.6****	**15.1****	**8.2**	**32.8**	**4.5***	**21.2****	**0.6****	218	327
	12.2	67.0	150	194	105	423	58.3	273	7.7		
B14 a42	**0.9**	**5.1**	**11.2****	**14.9****	**12.3**	**34.2**	**6.0**	**14.2**	**1.2**	110*	184**
	6.1	33.5	73.3	97.1	80.5	223	38.8	92.7	7.7		
B14 b1	**1.1****	**5.4**	**9.1***	**12.3**	**7.6**	**36.2**	**5.9**	**21.8****	**0.7****	191	266
	12.2	60.9	103	139	86.0	410	66.6	247	7.7		
B14 b2	**0.9**	**5.6**	**8.7**	**12.6**	**13.2**	**38.1**	**7.0**	**12.7**	**1.3**	173	295
	9.1	57.9	89.9	130	136	393	72.1	131	12.9		
B14 Mean	**0.9 (0.2)**	**5.3 (0.3)**	**8.9 (2.0)**	**12.5 (2.0)**	**11.6 (3.0)**	**38.0 (3.9)**	**6.1 (0.8)**	**15.8 (4.0)**	**1.0 (0.3)**	192 (41.2)	341 (117)
n = 7	10.0 (5.3)	73.4 (21.1)	133 (91.2)	175 (102)	142 (64.8)	550 (107)	74.9 (20.0)	241 (70.1)	12.6 (3.2)		

Table 4.5 continued.

Line	Products of TFA hydrolysis (mol%; μmol/ g AIR)									Total mass of identified products (mg/ g AIR)	Mass of hydrolysis residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA		
Ws	**0.8 (0.1)**	**6.7 (0.7)**	**7.2 (0.8)**	**10.6 (1.2)**	**11.3 (2.0)**	**40.4 (4.2)**	**6.2 (0.8)**	**15.4 (1.2)**	**1.4 (0.3)**	182 (28.2)	348 (52.0)
n = 6	8.9 (1.1)	72.6 (8.6)	77.8 (11.5)	114 (14.4)	121 (19.7)	444 (103)	68.3 (17.2)	167 (23.8)	15.5 (4.9)		
Col	**0.8 (0.2)**	**5.0 (0.3)**	**6.7 (0.8)**	**11.7* (1.1)**	**16.0** (3.4)**	**39.3 (3.5)**	**4.9** (0.4)**	**14.5 (1.5)**	**1.0 (0.2)**	173 (13.8)	380 (13.8)
n = 6	8.1 (1.5)	51.3 (5.3)	70.0 (11.1)	122 (12.6)	166 (39.6)	412 (58.0)	51.5 (6.0)	149.5 (12.8)	10.6 (2.3)		
B16-1	**0.8**	**5.0***	**6.6**	**9.8**	**6.7**	**48.3**	**5.7**	**16.1**	**1.1**	193	407
	9.1	57.9	76.6	114	77.7	563	66.6	188	12.9		
B16-5	**0.8**	**4.8***	**9.2***	**11.7**	**4.8****	**43.4**	**4.6**	**19.8****	**0.9**	182	279
	9.1	51.8	99.9	128	52.7	473	49.9	216	10.3		
B16 a1	**0.7**	**5.0**	**7.8**	**10.2**	**10.9**	**42.1**	**6.9**	**15.1**	**1.4**	222	387
	9.1	67.0	103	136	144	560	91.6	201	18.0		
B16 a6	**0.9**	**4.9***	**7.0**	**9.0**	**9.7**	**41.4**	**7.4**	**18.6****	**1.2**	177	380
	9.1	51.8	73.2	94.3	103	436	77.7	196	12.9		
B16 b1	**0.8**	**5.2**	**6.8**	**9.8**	**13.0**	**42.7**	**7.5**	**13.1**	**1.1**	245*	407
	12.2	76.1	99.9	144	191	630	111	193	15.5		
B16 b6	**0.9**	**5.2**	**7.4**	**11.4**	**14.5**	**40.1**	**4.1***	**15.9**	**0.6****	294**	388
	15.2	91.4	130	200	255	703	72.1	278	10.3		
B16 c6	**0.8**	**5.1**	**8.4**	**11.3**	**10.4**	**41.3**	**5.0**	**16.7**	**0.8***	257*	374
	12.2	79.2	130	175	161	636	77.7	258	12.9		
B16 d5	**1.3****	**5.9**	**18.5****	**21.7****	**7.9**	**20.8****	**2.8****	**20.7****	**0.4****	324**	306
	24.4	113	353	413	150	396	52.7	394	7.7		
B16 Mean	**0.9 (0.2)**	**5.1 (0.4)**	**9.0 (4.0)**	**11.9 (4.1)**	**9.7 (3.2)**	**40.0 (8.1)**	**5.5 (1.7)**	**17.0 (2.5)**	**0.9 (0.3)**	236 (41.2)	366 (47.4)
n = 8	12.6 (5.3)	73.5 (21.1)	133 (91.2)	175 (102)	142 (64.8)	550 (107)	74.9 (20.0)	241 (70.2)	12.6 (3.2)		

Table 4.5 continued.

Line	Products of TFA hydrolysis (mol%; μmol/ g AIR) Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA	Total mass of identified products (mg/ g AIR)	Mass of hydrolysis residue (mg/ g AIR)
Ws	**0.8 (0.1)**	**6.7 (0.7)**	**7.2 (0.8)**	**10.6 (1.2)**	**11.3 (2.0)**	**40.4 (4.2)**	**6.2 (0.8)**	**15.4 (1.2)**	**1.4 (0.3)**	182 (28.2)	348 (52.0)
n = 6	8.9 (1.1)	72.6 (8.6)	77.8 (11.5)	114 (14.4)	121 (19.7)	444 (103)	68.3 (17.2)	167 (23.8)	15.5 (4.9)		
Col	**0.8 (0.2)**	**5.0 (0.3)**	**6.7 (0.8)**	**11.7* (1.1)**	**16.0** (3.4)**	**39.3 (3.5)**	**4.9** (0.4)**	**14.5 (1.5)**	**1.0 (0.2)**	173 (13.8)	380 (13.8)
n = 6	8.1 (1.5)	51.3 (5.3)	70.0 (11.1)	122 (12.6)	166 (39.6)	412 (58.0)	51.5 (6.0)	149.5 (12.8)	10.6 (2.3)		
B17-4	**1.2****	**6.2**	**11.2****	**15.0****	**7.6**	**31.3***	**4.8**	**22.3****	**0.3****	166	255
	12.2	60.9	110	147	74.9	306	47.2	219	2.6		
B17-5	**0.8**	**4.5****	**6.6**	**9.6**	**6.5***	**48.1**	**5.5**	**16.9**	**1.6**	134	288
	6.1	36.5	53.3	77.7	52.7	389.7	44.4	137	12.9		
B17 a2	**0.7**	**4.9***	**8.1**	**11.9**	**7.8**	**42.2**	**5.5**	**18.1***	**0.8***	219	354
	9.1	63.9	107	155	103	553	72.1	237	10.3		
B17 b1	**0.9**	**4.9***	**8.7**	**10.6**	**9.2**	**42.5**	**5.7**	**16.3**	**1.3**	236	349
	12.2	70.0	123	150	130	603	80.4	232	18.0		
B17 b2	**0.8**	**5.5**	**10.9****	**15.4****	**11.6**	**34.4**	**5.5**	**15.0**	**0.9**	185	314
	9.1	60.9	120	169	128	380	61.0	165	10.3		
B17 c1	**0.8**	**5.2**	**6.6**	**10.3**	**8.8**	**46.7**	**5.1**	**15.7**	**0.7****	125	383
	6.1	39.6	50.0	77.7	66.6	353	38.8	118	5.2		
B17 d2	**0.7**	**5.0***	**7.8**	**10.8**	**12.9**	**43.0**	**6.5**	**12.6**	**0.8***	214	459
	9.1	63.9	99.9	138.7	166.5	553	83.2	162	10.3		
B17 Mean	**0.8 (0.2)**	**5.2 (0.6)**	**8.6 (1.9)**	**11.9 (2.3)**	**9.2 (2.3)**	**41.2 (6.2)**	**5.5 (0.5)**	**16.7 (3.0)**	**0.9(0.4)**	183 (42.9)	343 (66.8)
n = 7	9.1 (2.5)	56.6 (13.0)	94.7 (30.4)	131 (37.4)	103 (40.9)	448 (118)	61.0 (18.1)	181 (47.7)	9.9 (5.0)		

Table 4.5 continued.

Line	Products of TFA hydrolysis (mol%; µmol/ g AIR)									Total mass of identified products (mg/ g AIR)	Mass of hydrolysis residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA		
Ws	**0.8 (0.1)**	**6.7 (0.7)**	**7.2 (0.8)**	**10.6 (1.2)**	**11.3 (2.0)**	**40.4 (4.2)**	**6.2 (0.8)**	**15.4 (1.2)**	**1.4 (0.3)**	182 (28.2)	348 (52.0)
n = 6	8.9 (1.1)	72.6 (8.6)	77.8 (11.5)	114 (14.4)	121 (19.7)	444 (103)	68.3 (17.2)	167 (23.8)	15.5 (4.9)		
Col	**0.8 (0.2)**	**5.0 (0.3)**	**6.7 (0.8)**	**11.7* (1.1)**	**16.0** (3.4)**	**39.3 (3.5)**	**4.9** (0.4)**	**14.5 (1.5)**	**1.0 (0.2)**	173 (13.8)	380 (13.8)
n = 6	8.1 (1.5)	51.3 (5.3)	70.0 (11.1)	122 (12.6)	166 (39.6)	412 (58.0)	51.5 (6.0)	149.5 (12.8)	10.6 (2.3)		
B24-1	**1.3****	**8.0**	**16.3****	**27.0****	**14.9**	**13.3****	**6.3**	**11.6****	**1.4**	134	136**
	10.1	62.8	127	211	116	104	49.1	91.2	10.8		
B24-6	**1.2****	**6.8**	**11.7****	**18.2****	**13.5**	**29.0****	**7.1**	**11.5****	**1.1**	122	222*
	8.4	49.3	84.6	132	97.6	209	51.0	83.0	7.9		
B24 a4	**0.9**	**5.8**	**8.0**	**12.7**	**13.8**	**39.6**	**8.2**	**9.2****	**1.8**	146	271
	8.0	50.8	70.6	112	121	349	72.3	80.8	15.6		
B24 c4	**0.8**	**5.5**	**10.2****	**14.1****	**12.0**	**34.6**	**6.9**	**14.8**	**1.0**	199	205*
	9.9	65.2	121	167	142	410	81.3	175	12.1		
B24 d3	**0.7**	**4.6***	**7.4**	**10.1**	**9.9**	**44.9**	**5.2**	**16.1**	**1.1**	164	491*
	7.2	45.7	73.5	99.7	97.5	445	51.6	159	11.2		
B24 d4	**1.2****	**6.7**	**14.9****	**18.5****	**7.9**	**25.0****	**4.9**	**20.4****	**0.5****	171	321
	12.2	66.9	150	186	79.5	251	49.2	205	5.3		
B24 Mean	**1.0 (0.2)**	**6.2 (1.2)**	**11.4 (3.6)**	**16.8 (6.0)**	**12.0 (2.6)**	**31.1 (11.3)**	**6.4 (1.2)**	**13.9 (4.0)**	**1.2 (0.4)**	156 (27.7)	274 (123)
n = 6	9.3 (1.8)	56.8 (9.2)	105 (32.7)	151 (44.0)	109 (22.1)	295 (130)	59.1 (14.1)	132 (54.0)	10.5 (3.5)		

Table 4.5 continued.

Line	Products of TFA hydrolysis (mol%; µmol/ g AIR)									Total mass of identified products (mg/ g AIR)	Mass of hydrolysis residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA		
Ws	**0.8 (0.1)**	**6.7 (0.7)**	**7.2 (0.8)**	**10.6 (1.2)**	**11.3 (2.0)**	**40.4 (4.2)**	**6.2 (0.8)**	**15.4 (1.2)**	**1.4 (0.3)**	182 (28.2)	348 (52.0)
n = 6	8.9 (1.1)	72.6 (8.6)	77.8 (11.5)	114 (14.4)	121 (19.7)	444 (103)	68.3 (17.2)	167 (23.8)	15.5 (4.9)		
Col	**0.8 (0.2)**	**5.0 (0.3)**	**6.7 (0.8)**	**11.7* (1.1)**	**16.0** (3.4)**	**39.3 (3.5)**	**4.9** (0.4)**	**14.5 (1.5)**	**1.0 (0.2)**	173 (13.8)	380 (13.8)
n = 6	8.1 (1.5)	51.3 (5.3)	70.0 (11.1)	122 (12.6)	166 (39.6)	412 (58.0)	51.5 (6.0)	149.5 (12.8)	10.6 (2.3)		
B27 a1	**1.1****	**6.5**	**12.9****	**16.5****	**12.7**	**26.8****	**6.0**	**16.6**	**0.9**	129	224*
	8.3	49.3	98.1	126	96.8	204	45.5	126	6.9		
B27 b1	**1.3****	**6.6**	**18.8****	**20.6****	**8.8**	**19.2****	**4.7**	**19.5****	**0.7****	55.9**	219*
	4.1	21.6	61.6	67.6	28.9	63.1	15.4	63.9	2.3		
B27 c2	**0.9**	**5.5**	**8.2**	**11.6**	**13.7**	**38.3**	**6.8**	**13.9**	**1.1**	96.0**	256
	5.1	31.4	47.1	66.8	78.3	220	39.2	79.9	6.4		
B27 c6	**1.0**	**6.0**	**12.2****	**14.2****	**7.8**	**34.9**	**5.6**	**17.8***	**0.6****	153	273
	8.8	54.4	111	129	70.8	318	51.1	162	5.2		
B27 d1	**0.9**	**5.6**	**12.7****	**15.4****	**7.9**	**36.2**	**4.9**	**15.8**	**0.7***	169	287
	8.8	56.8	129	156	80.1	367	49.9	160	7.8		
B27 Mean	**1.0 (0.2)**	**6.0 (0.5)**	**13.0 (3.8)**	**15.7 (3.3)**	**10.2 (2.8)**	**31.1 (7.9)**	**5.6 (0.9)**	**16.7 (2.1)**	**0.8 (0.2)**	121 (45.4)	252 (29.5)
n = 5	7.0 (2.3)	42.7 (15.4)	89.4 (34.1)	109 (40.1)	71.0 (25.4)	234 (117)	40.2 (14.6)	118 (45.3)	5.7 (2.1)		

Table 4.5 continued.

Line	Products of TFA hydrolysis (mol%; µmol/ g AIR)									Total mass of identified products (mg/ g AIR)	Mass of hydrolysis residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA		
Ws	**0.8 (0.1)**	**6.7 (0.7)**	**7.2 (0.8)**	**10.6 (1.2)**	**11.3 (2.0)**	**40.4 (4.2)**	**6.2 (0.8)**	**15.4 (1.2)**	**1.4 (0.3)**	182 (28.2)	348 (52.0)
n = 6	8.9 (1.1)	72.6 (8.6)	77.8 (11.5)	114 (14.4)	121 (19.7)	444 (103)	68.3 (17.2)	167 (23.8)	15.5 (4.9)		
Col	**0.8 (0.2)**	**5.0 (0.3)**	**6.7 (0.8)**	**11.7* (1.1)**	**16.0** (3.4)**	**39.3 (3.5)**	**4.9** (0.4)**	**14.5 (1.5)**	**1.0 (0.2)**	173 (13.8)	380 (13.8)
n = 6	8.1 (1.5)	51.3 (5.3)	70.0 (11.1)	122 (12.6)	166 (39.6)	412 (58.0)	51.5 (6.0)	149.5 (12.8)	10.6 (2.3)		
B34-2	**0.9**	**4.9***	**7.3****	**11.8**	**8.1**	**40.2**	**6.4**	**19.9****	**0.7****	155	304
	7.9	45.1	67.4	109	74.8	371	58.9	184	6.4		
B34-3	**1.4****	**6.8**	**15.6****	**21.2****	**6.8**	**15.0****	**4.8**	**28.3****	**0.2****	87.8**	162**
	6.9	34.5	79.0	107	34.5	76.0	24.0	143	0.9		
B34 a4	**1.0***	**6.5**	**9.8****	**14.5****	**15.8***	**30.9***	**5.1**	**15.9**	**0.5****	166	304
	10.1	63.6	96.0	142	146	304	50.7	157	5.3		
B34 c4	**0.9**	**5.4**	**7.4**	**11.0**	**9.2**	**43.0**	**5.5**	**16.9**	**0.6****	210	410
	11.4	67.7	93.1	139	116	542	69.7	213	7.8		
B34 c5	**0.6**	**4.2****	**7.9**	**9.7**	**6.3***	**51.3***	**5.3**	**13.9**	**0.7****	173	457
	6.7	44.3	83.4	103	66.8	542	55.8	147	7.8		
B34 d3	**0.9**	**5.7**	**9.7****	**13.6****	**12.9**	**35.6**	**6.4**	**14.7**	**0.7****	243*	385
	12.4	81.8	140	197	187	515	92.1	212	9.6		
B34 Mean	**0.9 (0.2)**	**5.6 (1.0)**	**9.6 (3.1)**	**13.6 (4.1)**	**9.9 (3.8)**	**36.0 (12.4)**	**5.6 (0.7)**	**18.3 (5.3)**	**0.6 (0.2)**	173 (52.6)	337 (105)
n = 6	9.2 (2.4)	56.2 (17.8)	93.1 (25.1)	133 (35.8)	106 (57.9)	392 (184)	58.5 (22.4)	176 (31.9)	6.3 (3.0)		

Table 4.5 continued.

Line	Products of TFA hydrolysis (mol%; µmol/ g AIR)									Total mass of identified products (mg/ g AIR)	Mass of hydrolysis residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA		
Ws	**0.8 (0.1)**	**6.7 (0.7)**	**7.2 (0.8)**	**10.6 (1.2)**	**11.3 (2.0)**	**40.4 (4.2)**	**6.2 (0.8)**	**15.4 (1.2)**	**1.4 (0.3)**	182 (28.2)	348 (52.0)
n = 6	8.9 (1.1)	72.6 (8.6)	77.8 (11.5)	114 (14.4)	121 (19.7)	444 (103)	68.3 (17.2)	167 (23.8)	15.5 (4.9)		
Col	**0.8 (0.2)**	**5.0 (0.3)**	**6.7 (0.8)**	**11.7* (1.1)**	**16.0** (3.4)**	**39.3 (3.5)**	**4.9** (0.4)**	**14.5 (1.5)**	**1.0 (0.2)**	173 (13.8)	380 (13.8)
n = 6	8.1 (1.5)	51.3 (5.3)	70.0 (11.1)	122 (12.6)	166 (39.6)	412 (58.0)	51.5 (6.0)	149.5 (12.8)	10.6 (2.3)		
B52-1	**1.3****	**7.6**	**7.6**	**17.7****	**11.4**	**25.5****	**6.4**	**17.2**	**0.6****	67.7**	267
	5.0	30.5	49.1	70.7	45.6	102	25.4	68.4	2.5		
B52-4	**1.0***	**5.5**	**5.5**	**14.9****	**8.0**	**31.9***	**6.7**	**20.2****	**0.7****	185	289
	10.8	60.6	122	162	87.8	249	73.4	221	7.2		
B52-5	**0.7**	**4.6***	**4.6****	**11.5**	**11.1**	**41.4**	**7.8**	**13.2**	**1.2**	170	359
	7.2	47.5	86.2	117	114	424	80.1	135	12.1		
B52 a3	**1.0**	**6.3**	**6.3**	**16.1****	**11.6**	**29.5****	**6.6**	**17.4**	**0.6****	82.7**	368
	4.8	30.8	53.4	78.8	56.8	114	32.1	85.1	2.7		
B52 a5	**1.1****	**6.6**	**6.6**	**15.8****	**11.8**	**28.4****	**6.4**	**17.1**	**0.8***	128	278
	8.2	50.4	89.8	120	89.7	215	48.8	130	6.3		
B52 c4	**1.0***	**5.8**	**5.8**	**17.7****	**6.9**	**28.0****	**5.8**	**18.9****	**0.6****	173	262
	10.2	59.2	157	182	71.0	287	58.9	194	5.7		
B52 c5	**1.0***	**6.1**	**6.1**	**13.5***	**10.9**	**33.2**	**7.1**	**17.9***	**0.7****	146	328
	8.6	52.7	82.9	117	94.8	289	61.9	156	5.8		
B52 Mean	**1.0 (0.2)**	**6.1 (0.9)**	**6.1 (0.9)**	**15.3 (2.3)**	**10.3 (2.0)**	**31.1 (5.2)**	**6.7 (0.7)**	**17.4 (2.2)**	**0.7 (0.2)**	136 (45.8)	307 (44.1)
n = 7	7.8 (2.3)	47.4 (12.4)	91.5 (37.8)	121 (40.3)	79.9 (23.5)	258 (113)	54.4 (20.3)	141 (54.7)	6.0 (3.2)		

Table 4.5 continued.

Line	Products of TFA hydrolysis (mol%; µmol/ g AIR)									Total mass of identified products (mg/ g AIR)	Mass of hydrolysis residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA		
Ws	**0.8 (0.1)**	**6.7 (0.7)**	**7.2 (0.8)**	**10.6 (1.2)**	**11.3 (2.0)**	**40.4 (4.2)**	**6.2 (0.8)**	**15.4 (1.2)**	**1.4 (0.3)**	182 (28.2)	348 (52.0)
n = 6	8.9 (1.1)	72.6 (8.6)	77.8 (11.5)	114 (14.4)	121 (19.7)	444 (103)	68.3 (17.2)	167 (23.8)	15.5 (4.9)		
Col	**0.8 (0.2)**	**5.0 (0.3)**	**6.7 (0.8)**	**11.7* (1.1)**	**16.0** (3.4)**	**39.3 (3.5)**	**4.9** (0.4)**	**14.5 (1.5)**	**1.0 (0.2)**	173 (13.8)	380 (13.8)
n = 6	8.1 (1.5)	51.3 (5.3)	70.0 (11.1)	122 (12.6)	166 (39.6)	412 (58.0)	51.5 (6.0)	149.5 (12.8)	10.6 (2.3)		
B58-4	**0.9**	**5.4**	**7.3**	**12.8**	**8.0**	**38.7**	**7.5**	**18.6****	**0.8***	81.5**	301
	4.4	26.0	35.6	61.9	38.9	188	36.4	90.2	4.0		
B58-7	**1.0**	**5.9**	**9.8****	**13.5***	**5.3****	**37.7**	**5.0**	**21.6****	**0.4****	164	252
	9.6	58.0	95.7	132	51.8	368	48.5	211	3.6		
B58 b2	**1.2****	**6.5**	**14.3****	**20.6****	**12.7**	**22.4****	**4.5***	**17.0**	**0.9***	61.0**	283
	4.3	23.3	51.2	73.7	45.6	80.1	15.9	60.7	3.2		
B58 c1	**0.8**	**5.3**	**9.1***	**12.7**	**9.5**	**39.4**	**5.0**	**17.0**	**1.3**	102**	358
	4.8	32.4	55.4	77.7	57.7	240	30.6	103	7.7		
B58 c2	**1.3****	**6.8**	**16.0****	**22.1****	**10.1**	**19.5****	**5.9**	**17.8***	**0.5****	152	232*
	11.3	60.3	142	197	90.1	173	52.2	158	4.1		
B58 d3	**1.4****	**7.5**	**19.0****	**25.2****	**10.1**	**13.1****	**6.1**	**17.4**	**0.2****	125	228*
	10.0	54.7	139	184	74.1	95.4	44.5	127	1.7		
B58 Mean	**1.1 (0.2)**	**6.2 (0.9)**	**12.6 (4.5)**	**17.8 (5.5)**	**9.3 (2.5)**	**28.5 (11.5)**	**5.7 (1.1)**	**18.2 (1.8)**	**0.7 (0.4)**	114 (40.2)	276 (49.4)
n = 6	7.4 (3.2)	42.4 (17.0)	86.4 (46.3)	121 (59.1)	59.7 (19.1)	191 (105)	38.0 (13.4)	125 (53.4)	4.1 (2.0)		

4.3.3 Driselase digestion

The Driselase digestion products were separated on a Dionex HPLC (Section 2.2.5.3). Initially HPLC method 1 (Figure 2.1) was used, which separated the monosaccharides and uronic acids. However, this method only partially separated the disaccharide products, so method 2 (Figure 2.2) was developed to separate these compounds. Figures 4.7 and 4.8 show the Driselase digestion products of the AIR from *A. thaliana* ecotype Ws tissue.

The proportion of Driselase digestion products (as mol%), the total mass of products and the mass of the digestion residues for each of the nine lines, plus Ws and Columbia, are shown in Table 4.6. Table 4.6 gives the proportions (mol%) and the yields (μmol/ g AIR) of the screen products. The use of proportions removes variability due to differences in the proportion of the AIR solubilised and allows comparisons to be made between *A. thaliana* lines. The yields of the products allow comparisons between the results of the two screens for a particular *A. thaliana* line. As Driselase can undergo slight autolysis upon incubation, a Driselase-only control was also analysed by HPLC (Figures 4.7c and 4.8c; Table 4.6). The values shown for the screen products of the *A. thaliana* lines (Table 4.6) have had the concentrations of the Driselase-only products subtracted from them.

4.3.3.1 Digestion residue

The residue of the AIR remaining after Driselase digestion was washed, dried under a vacuum and weighed. A difference from Ws in the mass of the residue would imply that the proportion of the AIR that was Driselase-susceptible had altered.

The digestion residues of Ws and Columbia did not have significantly different masses (Table 4.6). The Columbia residue was only 4% heavier than the Ws residue. Individuals from each of the Feldmann lines had residues that were significantly different in mass from Ws. The mass of the

residues ranged from 48% (B24-4) to 147% (B14 h) of the mass of the Ws residues.

4.3.3.2 Total mass of identified carbohydrate digestion products

The total mass of identified carbohydrate products is a measure of the proportion of Driselase-susceptible polysaccharides present in the AIR. In Columbia the mass of identified Driselase-susceptible polysaccharides was approximately the same as in Ws (Table 4.6).

None of the Feldmann lines showed any significant differences from Ws in the mass of the total identified products. The total mass of identified products ranged from 48% less (B24 j) to 64% (B24-4) more than in Ws (Table 4.6).

4.3.3.3 Identified carbohydrate digestion products

***A. thaliana* ecotype Ws**

The major components of the Driselase digest of Ws were glucose and galacturonic acid. Together they accounted for 63 mol% of the digestion products (Table 4.6). The large proportion of glucose in the digest is due primarily to the breakdown of cellulose by Driselase. Xyloglucan and the rhamnogalacturonans also contributed to the amount of glucose present (Driselase does not efficiently break down starch). The galacturonic acid in the digest is derived from the pectic polysaccharides in the AIR.

Arabinose, galactose, isoprimeverose and xylobiose together accounted for a further 29 mol% of the identified products. Isoprimeverose and xylobiose are products of the Driselase digestion of xyloglucan and xylan, respectively. Driselase contains α-L-fucosidase, β-D-galactosidase and endo-(1→4)-β-glucanase (which cleaves the glucan backbone at the reducing end of unsubstituted β-glucose residues) but no α-xylosidase so xyloglucan is broken down into monosaccharides and the disaccharide isoprimeverose (D-xylosyl-α-(1→6)-D-glucose).

Xylobiose (D-xylosyl-β-(1→4)-D-xylose) is a partial breakdown product of xylan. Driselase contains β-D-xylosidase activity, α-L-arabinosidase, α-L-fucosidase and also xylanase (endo-β-(1→4)-D-xylanase), but no α-D-glucuronidase activity. Therefore xylan is broken down to xylobiose, xylose, arabinose, fucose and di- and trisaccharides of (methyl)glucuronic acid and xylose (see Section 3.1.3.4).

Arabinose and galactose can be found in the cell wall in arabinogalactans, and are both components of the side chains of glycoproteins and the rhamnogalacturonans. Galactose can also be found in xyloglucan and arabinose in xylan. Mannose is a component of the oligosaccharide chain of glycoproteins, as well as being present in galacto- and glucomannans.

The proportion of free xylose in the digestion products was relatively low. This was due to the majority of the xylose from the backbone of xylan being present in the form of xylobiose (and also possibly in the unidentified products, Section 4.3.3.4). The free xylose in the digest is primarily derived from the side chains of xylan, with a small amount from rhamnogalacturonans. Rhamnose was also present in the digest in quite low amounts; it is derived from the pectic polysaccharides.

Fucose and glucuronic acid were both minor components of the digest, with the proportion of these sugars being 0.9 mol% and 0.3 mol%, respectively. Fucose is found in the side chains of xyloglucan and rhamnogalacturonans, and glucuronic acid is present in xylan and rhamnogalacturonans. Glucuronic acid is linked by β-glycosyl bonds in rhamnogalacturonan II and in xylan by α-glycosyl bonds. There is no α-glucuronidase activity in Driselase (see above and Section 3.1.3.4) therefore the glucuronic acid in the Driselase products will be derived from rhamnogalacturonan-II.

***A. thaliana* ecotype Columbia**

The *A. thaliana* ecotypes Columbia and Ws, while having approximately the same product profiles, did have some significant differences (Table 4.6). Columbia had more glucose (114% of the Ws mean) but less fucose (75%), arabinose (87%), xylose (77%) and mannose (62%). The proportions of isoprimeverose and xylobiose were not significantly different from those of Ws, being 95% and 84% of the Ws values, respectively.

T-DNA tagged lines

All of the nine Feldmann lines showed significant differences from the parental line (Ws) in the composition of the Driselase digestion products (Table 4.6). Individuals from each of the Feldmann lines showed significant differences from Ws in the proportion of the major digestion product, glucose, and the majority of lines (seven out of nine) had over 50% of the individuals analysed showing the significant difference. The proportion of glucose ranged from 24% less (B52 b) to 38% more (B24 h) than in Ws. The majority of these significant differences from Ws were increases in the proportion of glucose.

The proportion of the digestion products that was galacturonic acid varied from 10% (B16 a1) to 139% (B34 f) of that in Ws (Table 4.6). The majority of significant differences were decreases compared to Ws, with only three individuals having a significantly increased proportion of galacturonic acid (B14-h [129%], B27 g [131%] and B34 f).

Individuals from eight of the Feldmann lines had a proportion of rhamnose that was significantly different from Ws. The proportion of rhamnose in these lines varied from 53% (B34 a5) to 294% (B52 b) of that for Ws. Rhamnose and galacturonic acid are the major components of the pectic polysaccharides. There did not appear to be any correlation between the increases and decreases in rhamnose and galacturonic acid.

The proportion of the identified carbohydrate products that was arabinose varied from 58% less (B27 f) to 58% more (B17 b2) than Ws. There were significant differences from Ws in the proportion of arabinose in eight of the Feldmann lines.

Galactose was significantly lower than in Ws in individuals of all but one of the screened lines. The proportion of the digests that was galactose varied from 52% less (B52 b) to 13% more (B52 a3) than in Ws.

All of the lines had individuals with significantly different proportions of isoprimeverose and/ or xylobiose in the digest. For isoprimeverose, five of the lines (B5, B14, B16, B34 and B52) and, for xylobiose, eight of the lines (all except B17) had at least 50% of the individuals analysed showing the significant difference. The range of proportions for isoprimeverose was from 46% (B17 b2) to 200% (B14 j) of that for Ws; for xylobiose the range was from 32% (B27 h) to 232% (B5 b4, Table 4.6) of that for Ws.

The proportion of xylose in the digests varied from 59% less (B14-c) to 66% more (B16-4 and B52-1) than in Ws. Individuals from six of the Feldmann lines showed a significant difference from Ws. Only three of the significant differences were increases compared to Ws (B16-4, B52-1 and B52-5 [147% of Ws]).

Only four individuals (B14 c, B27 f, B27 g and B27 h) had significantly different proportions of mannose than in Ws. All these significant differences were decreases compared to Ws. The proportion of mannose in the Driselase products ranged from 28% (B27 g) to 120% (B5-2) of that for Ws.

All of the lines had individuals with significantly different proportions of fucose than Ws. However, fucose was a minor component of the screen products, making up less than 1.7 mol% of the identified products in all cases.

Glucuronic acid was present in the digests of individuals from all nine lines at significantly different proportions from Ws. The proportion of glucuronic acid ranged from 0% (in 16 individuals) to 267% (B16-4) of that in Ws. However, glucuronic acid is a minor component of the Driselase products, making up less than 1.0 mol% in all cases.

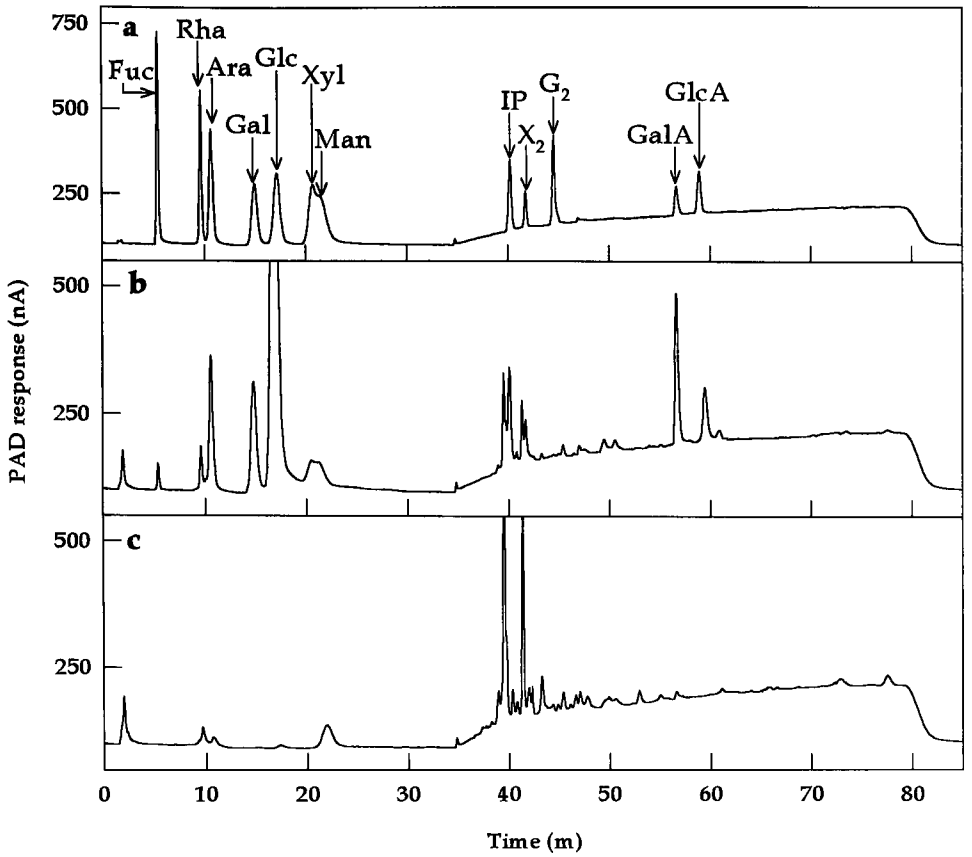

Figure 4.7 Driselase digestion products of the AIR of the flowering stem of *A. thaliana* ecotype Wassilewskija. The AIR was incubated with 0.5% Driselase (Section 2.2.4.1). The digestion products were separated on a Dionex HPLC using method 1 (Section 2.2.5.3). **(a)** A mix of standards, each at a concentration of 0.05 mg/ ml; **(b)** the Driselase digestion products of the AIR of the flowering stem of *A. thaliana* ecotype Wassilewskija (a 1 in 10 dilution of the original digest is shown); **(c)** a Driselase-only control (an undiluted digest is shown).

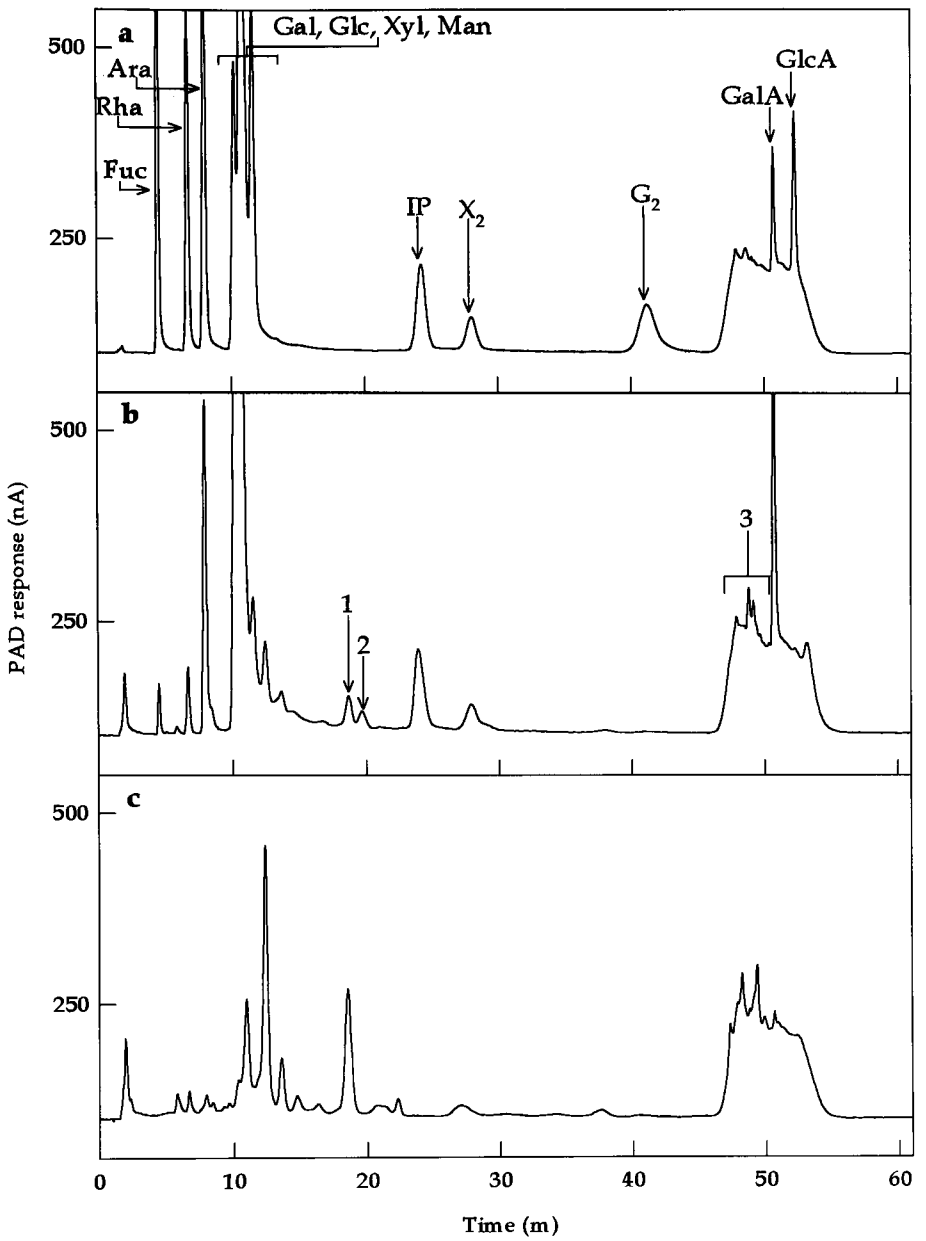

Figure 4.8 Driselase digestion products of the AIR of the flowering stem of *A. thaliana* ecotype Wassilewskija. Details as for Figure 4.7 except that HPLC method 2 was used and (b) shows a 1 in 10 dilution; 1-3 = unidentified products (Section 4.3.3.4).

Table 4.6 The Driselase digestion products of the AIR of *A. thaliana* lines. The AIR was incubated with 0.5% Driselase (Section 2.2.4.2). Digestion products were separated on a Dionex HPLC (Section 2.2.5.3). Chromatograms were calibrated using external standards and Dionex software. The figures shown for Ws and Columbia are means, with standard deviations in brackets. n = number of replicates; n.a. = not applicable; ** = probability of less than 0.05 that the line is from the same population as the parental line, Ws; * = probability of $0.05 < p < 0.10$ that the line is from the same population as the parental line, Ws (as calculated by Student's t distribution, Section 2.2.6). Control = Driselase-only incubation; products are given in µmol/ 25 ml 0.5% Driselase, which is equivalent to µmol/ g AIR; the values for the control products were subtracted from the screen products of the *A. thaliana* lines shown below.

Line	Products of Driselase digestion (mol%; µmol/ g AIR)											Total mass of identified products (mg/ g AIR)	Mass of digestion residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	IP	X_2	GalA	GlcA		
Ws	**0.9 (0.1)**	**1.7 (0.2)**	**8.0 (1.0)**	**9.6 (1.0)**	**41.4 (2.0)**	**3.2 (0.5)**	**2.5 (0.6)**	**5.6 (0.4)**	**5.7 (0.6)**	**21.2 (1.7)**	**0.3 (0.1)**	168 (58.7)	441 (39.6)
n = 6	6.9 (0.6)	12.8 (1.2)	60.4 (7.5)	73.0 (9.1)	316 (48.5)	24.3 (5.1)	18.7 (3.4)	42.1 (4.1)	43.2 (6.8)	162 (31.5)	2.2 (0.5)		
Col	**0.7** (0.1)**	**1.7 (0.2)**	**6.9** (1.0)**	**9.4 (0.5)**	**47.1** (2.4)**	**2.5** (0.4)**	**1.5** (0.1)**	**5.3 (0.6)**	**4.8 (1.5)**	**20.0 (3.1)**	**0.3 (0.1)**	169 (18.8)	460 (38.1)
n = 10	5.9 (0.8)	14.8 (1.5)	61.1 (12.9)	82.4 (8.0)	415 (44.6)	21.7 (4.7)	13.6 (1.7)	46.3 (7.8)	42.8 (16.7)	176 (34.9)	2.1 (1.0)		
B5-2	**0.9**	**1.9**	**12.1****	**8.8**	**45.7***	**2.7**	**3.0**	**7.9****	**4.5**	**12.4****	**0.2**	140	552*
	6.9	13.7	88.3	63.8	333	20.0	21.5	57.6	32.8	90.2	1.3		
B5-3′	**0.8**	**1.4**	**11.7****	**8.2**	**40.5**	**3.2**	**2.2**	**8.0****	**10.7****	**12.7****	**0.5***	169	343*
	6.9	12.2	99.1	69.4	344	27.5	18.8	68.0	91.2	108	3.9		
B5 a5	**0.7**	**1.5**	**9.1**	**7.5***	**41.6**	**3.3**	**2.3**	**7.3****	**9.8****	**16.4****	**0.4**	205	407
	7.6	15.3	94.1	77.7	430	34.1	24.3	76.0	101	170	3.9		
B5 a6	**0.9**	**2.1***	**11.8****	**6.8****	**35.6***	**4.2**	**2.3**	**7.4****	**10.8****	**17.7**	**0.5****	153	435
	6.9	16.0	90.8	52.7	275	32.5	18.1	56.8	83.3	137	3.9		
B5 b4	**0.7***	**1.3***	**11.2****	**8.6**	**39.9**	**3.7**	**1.6**	**6.5***	**13.2****	**13.0****	**0.4**	205	398
	6.9	13.0	116	88.8	411	38.3	16.7	66.4	136	134	3.9		
B5 c4	**0.7***	**1.4**	**10.6***	**7.8**	**39.2**	**3.1**	**1.4**	**6.5***	**13.1****	**15.7***	**0.6****	207	303**
	6.9	14.5	110	80.5	405	31.6	14.6	67.2	136	162	6.4		
B5 Mean	**0.8 (0.1)**	**1.6 (0.3)**	**11.1 (1.1)**	**7.9 (0.7)**	**40.4 (3.3)**	**3.4 (0.5)**	**2.1 (0.6)**	**7.3 (0.7)**	**10.3 (3.2)**	**14.7 (2.2)**	**0.4 (0.2)**	180 (29.7)	406 (85.9)
n = 6	7.0 (0.3)	14.1 (1.4)	99.7 (11.0)	72.2 (12.9)	366 (59.2)	30.7 (6.3)	19.0 (3.5)	65.8 (7.2)	96.6 (38.3)	134 (30.6)	3.9 (1.6)		
	(µmol/ 25 ml 0.5% Driselase)												
Control	0.0 (0.0)	10.1 (15.9)	0.5 (1.3)	0.5 (1.1)	0.4 (0.9)	0.3 (0.9)	0.8 (1.7)	0.8 (2.3)	0.1 (0.3)	1.0 (1.3)	0.0 (0.0)	2.6 (3.3)	n.a.
n = 12													

Table 4.6 continued.

Line	Products of Driselase digestion (mol%; μmol/ g AIR)											Total mass of identified products (mg/ g AIR)	Mass of digestion residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	IP	X_2	GalA	GlcA		
Ws	**0.9 (0.1)**	**1.7 (0.2)**	**8.0 (1.0)**	**9.6 (1.0)**	**41.4 (2.0)**	**3.2 (0.5)**	**2.5 (0.6)**	**5.6 (0.4)**	**5.7 (0.6)**	**21.2 (1.7)**	**0.3 (0.1)**	168 (58.7)	441 (39.6)
n = 6	6.9 (0.6)	12.8 (1.2)	60.4 (7.5)	73.0 (9.1)	316 (48.5)	24.3 (5.1)	18.7 (3.4)	42.1 (4.1)	43.2 (6.8)	162 (31.5)	2.2 (0.5)		
Col	**0.7** (0.1)**	**1.7 (0.2)**	**6.9** (1.0)**	**9.4 (0.5)**	**47.1** (2.4)**	**2.5** (0.4)**	**1.5** (0.1)**	**5.3 (0.6)**	**4.8 (1.5)**	**20.0 (3.1)**	**0.3 (0.1)**	169 (18.8)	460 (38.1)
n = 10	5.9 (0.8)	14.8 (1.5)	61.1 (12.9)	82.4 (8.0)	415 (44.6)	21.7 (4.7)	13.6 (1.7)	46.3 (7.8)	42.8 (16.7)	176 (34.9)	2.1 (1.0)		
B14-1	**0.7***	**1.8**	**8.0**	**7.7**	**42.9**	**1.7****	**1.6**	**7.1****	**5.1**	**23.2**	**0.1***	181	505
	6.1	16.8	74.1	72.1	400	15.9	14.6	66.4	47.8	216	1.3		
B14-2	**0.6****	**1.6**	**8.4**	**7.9**	**44.1**	**2.7**	**1.5**	**7.3****	**11.6****	**13.8****	**0.4**	189	478
	6.1	15.3	80.0	74.9	419	25.8	13.9	69.6	110	131	3.9		
B14-3	**0.6****	**1.4**	**7.3**	**6.4****	**40.9**	**2.9**	**1.5**	**6.7****	**10.4****	**21.7**	**0.3**	189	552*
	5.4	13.7	69.2	61.0	389	27.5	13.9	64.0	98.3	206	2.6		
B14-5	**0.6****	**1.8**	**9.1**	**9.0**	**47.7****	**1.9***	**1.5**	**8.1****	**7.4****	**12.6****	**0.3**	169	473
	5.4	15.3	78.3	77.7	411	16.7	12.5	69.6	63.8	108	2.6		
B14-6	**0.7***	**1.4**	**9.7**	**8.5**	**44.3**	**3.1**	**1.6**	**7.8****	**12.4****	**10.2****	**0.4**	223	303**
	7.6	16.0	108.3	94.3	494	34.2	18.0	86.5	138.2	113	3.9		
B14 c	**0.8**	**1.8**	**6.7**	**6.9****	**42.2**	**1.3****	**0.9***	**10.0****	**5.3**	**23.9**	**0.1***	191	516
	7.6	17.5	64.2	66.6	405	12.5	9.0	96.1	50.5	229	1.3		
B14 g	**0.7**	**1.6**	**8.8**	**8.9**	**41.4**	**2.5**	**2.0**	**8.7****	**6.8**	**17.8**	**0.6****	123	546*
	4.6	9.9	55.0	55.5	258	15.9	12.5	54.4	42.5	111	3.9		
B14 h	**0.7**	**2.0**	**5.3****	**6.3****	**41.7**	**1.8***	**1.7**	**9.1****	**4.0***	**27.4****	**0.0****	148	647**
	5.4	15.3	40.0	47.2	314	13.4	12.5	68.0	30.1	206	0.0		
B14 j	**1.1**	**3.2****	**7.1**	**8.6**	**46.7****	**2.6**	**1.7**	**11.2****	**6.8**	**10.8****	**0.2**	128	500
	6.9	20.6	45.8	55.5	300	16.7	11.1	72.0	43.4	69.6	1.3		
B14 Mean	**0.7 (0.2)**	**1.9 (0.5)**	**7.8 (1.4)**	**7.8 (1.1)**	**43.6 (2.4)**	**2.3 (0.6)**	**1.5 (0.3)**	**8.5 (4.5)**	**7.8 (3.0)**	**17.9 (6.4)**	**0.3 (0.2)**	172 (32.7)	502 (91.2)
n = 9	6.1 (1.1)	15.6 (2.9)	68.3 (20.5)	67.2 (14.3)	376 (72.6)	19.8 (7.5)	13.1 (2.5)	71.8 (12.4)	69.4 (37.1)	155 (59.4)	2.3 (1.4)		
	(μmol/ 25 ml 0.5% Driselase)												
Control	0.0 (0.0)	10.1 (15.9)	0.5 (1.3)	0.5 (1.1)	0.4 (0.9)	0.3 (0.9)	0.8 (1.7)	0.8 (2.3)	0.1 (0.3)	1.0 (1.3)	0.0 (0.0)	2.6 (3.3)	n.a.
n = 12													

Table 4.6 continued.

Line	Products of Driselase digestion (mol%; μmol/ g AIR) Fuc	Rha	Ara	Gal	Glc	Xyl	Man	IP	X_2	GalA	GlcA	Total mass of identified products (mg/ g AIR)	Mass of digestion residue (mg/ g AIR)
Ws	**0.9 (0.1)**	**1.7 (0.2)**	**8.0 (1.0)**	**9.6 (1.0)**	**41.4 (2.0)**	**3.2 (0.5)**	**2.5 (0.6)**	**5.6 (0.4)**	**5.7 (0.6)**	**21.2 (1.7)**	**0.3 (0.1)**	168 (58.7)	441 (39.6)
n = 6	6.9 (0.6)	12.8 (1.2)	60.4 (7.5)	73.0 (9.1)	316 (48.5)	24.3 (5.1)	18.7 (3.4)	42.1 (4.1)	43.2 (6.8)	162 (31.5)	2.2 (0.5)		
Col	**0.7** (0.1)**	**1.7 (0.2)**	**6.9** (1.0)**	**9.4 (0.5)**	**47.1** (2.4)**	**2.5** (0.4)**	**1.5** (0.1)**	**5.3 (0.6)**	**4.8 (1.5)**	**20.0 (3.1)**	**0.3 (0.1)**	169 (18.8)	460 (38.1)
n = 10	5.9 (0.8)	14.8 (1.5)	61.1 (12.9)	82.4 (8.0)	415 (44.6)	21.7 (4.7)	13.6 (1.7)	46.3 (7.8)	42.8 (16.7)	176 (34.9)	2.1 (1.0)		
B16-2	**0.6****	**1.0****	**9.0**	**7.3***	**39.5**	**3.4**	**1.5**	**5.8**	**6.5**	**19.7**	**0.4***	176	484
	4.6	9.2	79.1	63.8	347	30.0	13.2	51.2	56.7	173	3.9		
B16-4	**0.7***	**1.4**	**9.8**	**7.5***	**42.2**	**5.3****	**1.6**	**6.2**	**6.8**	**12.1****	**0.8****	269	335*
	8.3	17.5	123	94.3	530	66.6	20.1	77.6	85.9	152	10.3		
B16 a1	**0.9**	**2.1***	**12.5****	**9.0**	**48.9****	**4.0**	**2.2**	**6.0**	**6.6**	**2.1****	**0.2**	120	478
	5.4	13.0	77.5	55.5	302	25.0	13.9	36.8	40.8	12.9	1.3		
B16 b1	**0.6****	**1.7**	**8.3**	**6.9****	**39.5**	**2.7**	**1.9**	**6.8****	**7.5****	**17.7**	**0.3**	166	462
	5.4	15.2	73.3	61.0	350	24.2	16.6	60.0	66.4	157	2.6		
B16 c	**0.9**	**2.1***	**5.8***	**6.3****	**35.0****	**1.9***	**1.2**	**9.2****	**10.2****	**19.0**	**0.2**	121	462
	6.1	14.5	40.8	44.4	247	13.3	8.3	64.8	71.8	134	1.3		
B16 i	**0.8**	**2.1***	**5.4****	**6.1****	**32.7****	**1.9***	**1.5**	**8.8****	**9.8****	**22.4**	**0.5****	185	505
	8.4	22.1	56.7	63.8	341	20.0	15.3	92.1	102	234	5.2		
B16 Mean	**0.8 (0.2)**	**1.7 (0.5)**	**8.5 (2.6)**	**7.2 (1.0)**	**39.6 (5.7)**	**3.2 (1.3)**	**1.7 (0.4)**	**7.1 (1.5)**	**7.9 (1.7)**	**15.5 (7.4)**	**0.4 (0.2)**	173 (54.6)	454 (60.6)
n = 6	6.4 (1.6)	15.2 (4.4)	75.1 (27.8)	63.8 (16.6)	353 (95.2)	29.8 (18.9)	14.6 (3.9)	63.8 (19.5)	70.6 (21.5)	144 (72.8)	4.1 (3.4)		
	(μmol/ 25 ml 0.5% Driselase)												
Control	0.0 (0.0)	10.1 (15.9)	0.5 (1.3)	0.5 (1.1)	0.4 (0.9)	0.3 (0.9)	0.8 (1.7)	0.8 (2.3)	0.1 (0.3)	1.0 (1.3)	0.0 (0.0)	2.6 (3.3)	n.a.
n = 12													

Table 4.6 continued.

Line	Products of Driselase digestion (mol%; µmol/ g AIR) Fuc	Rha	Ara	Gal	Glc	Xyl	Man	IP	X_2	GalA	GlcA	Total mass of identified products (mg/ g AIR)	Mass of digestion residue (mg/ g AIR)
Ws	**0.9 (0.1)**	**1.7 (0.2)**	**8.0 (1.0)**	**9.6 (1.0)**	**41.4 (2.0)**	**3.2 (0.5)**	**2.5 (0.6)**	**5.6 (0.4)**	**5.7 (0.6)**	**21.2 (1.7)**	**0.3 (0.1)**	168 (58.7)	441 (39.6)
n = 6	6.9 (0.6)	12.8 (1.2)	60.4 (7.5)	73.0 (9.1)	316 (48.5)	24.3 (5.1)	18.7 (3.4)	42.1 (4.1)	43.2 (6.8)	162 (31.5)	2.2 (0.5)		
Col	**0.7** (0.1)**	**1.7 (0.2)**	**6.9** (1.0)**	**9.4 (0.5)**	**47.1** (2.4)**	**2.5** (0.4)**	**1.5** (0.1)**	**5.3 (0.6)**	**4.8 (1.5)**	**20.0 (3.1)**	**0.3 (0.1)**	169 (18.8)	460 (38.1)
n = 10	5.9 (0.8)	14.8 (1.5)	61.1 (12.9)	82.4 (8.0)	415 (44.6)	21.7 (4.7)	13.6 (1.7)	46.3 (7.8)	42.8 (16.7)	176 (34.9)	2.1 (1.0)		
B17 b1	**0.5****	**1.4**	**6.4**	**7.8**	**47.6****	**2.3**	**1.2**	**5.4**	**6.0**	**16.3****	**0.2**	159	495
	6.1	17.5	80.0	97.1	594	29.2	15.3	67.2	74.4	204	2.6		
B17 b2	**0.8**	**1.8**	**12.6****	**10.6**	**55.7****	**4.2**	**1.5**	**2.6****	**2.9****	**4.9****	**0.0****	224	212**
	5.4	12.2	85.8	72.1	377	28.3	10.4	17.6	19.5	33.5	0.0		
B17 c1	**0.7**	**2.2****	**10.3***	**9.5**	**53.3****	**2.6**	**1.7**	**5.1**	**5.7**	**4.2****	**0.0****	137	495
	5.4	16.0	75.8	69.4	391	19.2	12.5	37.6	41.6	30.9	0.0		
B17 c2	**0.5****	**1.3**	**6.4**	**8.1**	**44.3**	**2.2**	**1.4**	**5.4**	**6.0**	**19.5**	**0.2**	215	432
	6.1	17.5	83.3	105	580	28.3	18.0	70.4	78.0	255	2.6		
B17 d2	**0.6****	**1.5**	**8.3**	**9.0**	**42.1**	**2.4**	**1.3**	**6.7****	**7.4****	**14.1****	**0.3**	248	260**
	6.9	17.5	96.6	105	491	28.3	14.6	78.5	86.8	165	3.9		
B17 Mean	**0.6 (0.1)**	**1.6 (0.4)**	**8.8 (2.7)**	**9.0 (1.1)**	**48.6 (5.8)**	**2.7 (0.8)**	**1.4 (0.2)**	**5.0 (1.5)**	**5.6 (1.6)**	**11.8 (6.9)**	**0.1 (0.1)**	196 (46.8)	393 (114)
n = 5	6.0 (0.6)	16.1 (2.3)	84.3 (7.9)	89.9 (17.8)	487 (102)	26.6 (4.2)	14.1 (2.9)	54.3 (25.7)	60.1 (28.4)	138 (101)	1.8 (1.7)		
	(µmol/ 25 ml 0.5% Driselase)												
Control	0.0 (0.0)	10.1 (15.9)	0.5 (1.3)	0.5 (1.1)	0.4 (0.9)	0.3 (0.9)	0.8 (1.7)	0.8 (2.3)	0.1 (0.3)	1.0 (1.3)	0.0 (0.0)	2.6 (3.3)	n.a.
n = 12													

Table 4.6 continued.

Line	Products of Driselase digestion (mol%; μmol/ g AIR)											Total mass of identified products (mg/ g AIR)	Mass of digestion residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	IP	X_2	GalA	GlcA		
Ws	**0.9 (0.1)**	**1.7 (0.2)**	**8.0 (1.0)**	**9.6 (1.0)**	**41.4 (2.0)**	**3.2 (0.5)**	**2.5 (0.6)**	**5.6 (0.4)**	**5.7 (0.6)**	**21.2 (1.7)**	**0.3 (0.1)**	168 (58.7)	441 (39.6)
n = 6	6.9 (0.6)	12.8 (1.2)	60.4 (7.5)	73.0 (9.1)	316 (48.5)	24.3 (5.1)	18.7 (3.4)	42.1 (4.1)	43.2 (6.8)	162 (31.5)	2.2 (0.5)		
Col	**0.7** (0.1)**	**1.7 (0.2)**	**6.9** (1.0)**	**9.4 (0.5)**	**47.1** (2.4)**	**2.5** (0.4)**	**1.5** (0.1)**	**5.3 (0.6)**	**4.8 (1.5)**	**20.0 (3.1)**	**0.3 (0.1)**	169 (18.8)	460 (38.1)
n = 10	5.9 (0.8)	14.8 (1.5)	61.1 (12.9)	82.4 (8.0)	415 (44.6)	21.7 (4.7)	13.6 (1.7)	46.3 (7.8)	42.8 (16.7)	176 (34.9)	2.1 (1.0)		
B24 b	**0.9**	**2.3****	**5.4****	**8.2**	**50.1****	**2.2**	**2.3**	**5.2**	**2.4****	**20.8**	**0.2**	148	544*
	6.9	18.3	42.5	63.8	391	17.5	18.1	40.8	18.6	162	1.3		
B24 c	**1.0**	**2.7****	**6.5**	**8.2**	**50.2****	**2.4**	**2.3**	**6.0**	**2.6****	**18.2**	**0.0****	135	506
	6.9	19.1	45.8	58.3	355	16.7	16.0	42.4	18.6	129	0.0		
B24 f	**0.9**	**2.4****	**6.6**	**7.3***	**46.5***	**2.8**	**2.2**	**5.2**	**2.6****	**23.5**	**0.2**	152	624**
	6.9	19.1	52.5	58.3	372	22.5	17.3	41.6	20.4	188	1.3		
B24 g	**0.8**	**2.5****	**5.7***	**7.6***	**53.3****	**2.7**	**1.8**	**4.8**	**2.5****	**18.3**	**0.0****	151	629**
	6.1	19.8	45.8	61.0	427	21.7	14.6	38.4	20.4	147	0.0		
B24 h	**0.7**	**1.6**	**5.3****	**5.2****	**57.1****	**1.9***	**1.4**	**4.5***	**2.0****	**20.5**	**0.0****	221	486
	8.4	18.3	61.7	61.0	669	22.5	16.0	52.8	23.0	240	0.0		
B24 j	**1.5****	**4.0****	**9.6**	**9.1**	**56.2****	**3.8**	**2.7**	**6.6***	**4.3***	**2.2****	**0.0****	86.6	568**
	6.9	18.3	44.2	41.6	258	17.5	12.5	30.4	19.5	10.3	0.0		
B24-4	**0.7**	**1.4**	**10.3***	**10.2**	**41.5**	**2.5**	**1.3**	**6.3**	**7.6****	**17.9**	**0.4**	276	211**
	10.7	19.8	152	150	610	36.7	18.8	92.1	112	263	6.4		
B24 Mean	**0.9 (0.3)**	**2.4 (0.9)**	**7.1 (2.1)**	**8.0 (1.6)**	**50.7 (5.5)**	**2.6 (0.6)**	**2.0 (0.5)**	**5.5 (0.8)**	**3.4 (2.0)**	**17.3 (7.0)**	**0.1 (0.2)**	167 (62.1)	510 (143)
n = 7	7.5 (1.5)	18.9 (0.7)	63.4 (39.4)	70.6 (35.8)	440 (147)	22.1 (6.9)	16.2 (2.1)	48.4 (20.4)	33.3 (34.9)	163 (82.9)	1.3 (2.3)		
	(μmol/ 25 ml 0.5% Driselase)												
Control	0.0 (0.0)	10.1 (15.9)	0.5 (1.3)	0.5 (1.1)	0.4 (0.9)	0.3 (0.9)	0.8 (1.7)	0.8 (2.3)	0.1 (0.3)	1.0 (1.3)	0.0 (0.0)	2.6 (3.3)	n.a.
n = 12													

Table 4.6 continued.

Line	Products of Driselase digestion (mol%; μmol/ g AIR)											Total mass of identified products (mg/ g AIR)	Mass of digestion residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	IP	X_2	GalA	GlcA		
Ws	**0.9 (0.1)**	**1.7 (0.2)**	**8.0 (1.0)**	**9.6 (1.0)**	**41.4 (2.0)**	**3.2 (0.5)**	**2.5 (0.6)**	**5.6 (0.4)**	**5.7 (0.6)**	**21.2 (1.7)**	**0.3 (0.1)**	168 (58.7)	441 (39.6)
n = 6	6.9 (0.6)	12.8 (1.2)	60.4 (7.5)	73.0 (9.1)	316 (48.5)	24.3 (5.1)	18.7 (3.4)	42.1 (4.1)	43.2 (6.8)	162 (31.5)	2.2 (0.5)		
Col	**0.7** (0.1)**	**1.7 (0.2)**	**6.9** (1.0)**	**9.4 (0.5)**	**47.1** (2.4)**	**2.5** (0.4)**	**1.5** (0.1)**	**5.3 (0.6)**	**4.8 (1.5)**	**20.0 (3.1)**	**0.3 (0.1)**	169 (18.8)	460 (38.1)
n = 10	5.9 (0.8)	14.8 (1.5)	61.1 (12.9)	82.4 (8.0)	415 (44.6)	21.7 (4.7)	13.6 (1.7)	46.3 (7.8)	42.8 (16.7)	176 (34.9)	2.1 (1.0)		
B27-6	**0.7***	**1.4**	**9.7**	**9.0**	**43.1**	**2.9**	**1.6**	**5.0**	**7.1***	**19.2**	**0.4***	227	532*
	7.6	16.0	114	105	508	34.2	18.7	59.2	83.3	227	5.2		
B27 b	**0.8**	**2.0**	**5.7***	**6.5****	**55.9****	**2.7**	**1.6**	**5.8**	**2.4****	**16.7***	**0.0****	170	228**
	6.9	17.5	50.8	58.3	499	24.2	13.9	52.0	21.3	149	0.0		
B27 f	**0.5****	**1.3***	**3.4****	**6.2****	**56.8****	**1.6****	**0.8****	**3.9****	**1.9****	**23.7**	**0.1****	271	444
	6.9	18.3	48.3	88.8	813	22.5	11.1	56.0	26.6	340	1.3		
B27 g	**0.6****	**1.5**	**4.5****	**6.3****	**49.7****	**2.0***	**0.7****	**5.0**	**1.9****	**27.8****	**0.0****	217	581**
	6.9	17.5	51.6	72.1	566	22.5	8.3	56.8	21.3	317	0.0		
B27 h	**0.5****	**1.3***	**4.2****	**6.1****	**56.9****	**2.0***	**1.0***	**3.9****	**1.8****	**22.3**	**0.0****	205	591**
	5.4	13.7	45.8	66.6	619	21.7	11.1	42.4	19.5	242	0.0		
B27 Mean	**0.6 (0.1)**	**1.5 (0.3)**	**5.5 (2.5)**	**6.8 (1.2)**	**52.5 (6.0)**	**2.2 (0.6)**	**1.1 (0.4)**	**4.7 (0.8)**	**3.0 (2.3)**	**22.0 (4.3)**	**0.1 (0.2)**	218 (36.6)	475 (150)
n = 5	6.7 (0.8)	16.6 (1.8)	62.1 (29.1)	78.2 (18.8)	601 (128)	25.0 (5.2)	12.6 (3.9)	53.3 (6.6)	34.4 (27.5)	255 (76.1)	1.3 (2.3)		
	(μmol/ 25 ml 0.5% Driselase)												
Control	0.0 (0.0)	10.1 (15.9)	0.5 (1.3)	0.5 (1.1)	0.4 (0.9)	0.3 (0.9)	0.8 (1.7)	0.8 (2.3)	0.1 (0.3)	1.0 (1.3)	0.0 (0.0)	2.6 (3.3)	n.a.
n = 12													

Table 4.6 continued.

Line	Products of Driselase digestion (mol%; μmol/ g AIR) Fuc	Rha	Ara	Gal	Glc	Xyl	Man	IP	X_2	GalA	GlcA	Total mass of identified products (mg/ g AIR)	Mass of digestion residue (mg/ g AIR)
Ws	**0.9 (0.1)**	**1.7 (0.2)**	**8.0 (1.0)**	**9.6 (1.0)**	**41.4 (2.0)**	**3.2 (0.5)**	**2.5 (0.6)**	**5.6 (0.4)**	**5.7 (0.6)**	**21.2 (1.7)**	**0.3 (0.1)**	168 (58.7)	441 (39.6)
n = 6	6.9 (0.6)	12.8 (1.2)	60.4 (7.5)	73.0 (9.1)	316 (48.5)	24.3 (5.1)	18.7 (3.4)	42.1 (4.1)	43.2 (6.8)	162 (31.5)	2.2 (0.5)		
Col	**0.7** (0.1)**	**1.7 (0.2)**	**6.9** (1.0)**	**9.4 (0.5)**	**47.1** (2.4)**	**2.5** (0.4)**	**1.5** (0.1)**	**5.3 (0.6)**	**4.8 (1.5)**	**20.0 (3.1)**	**0.3 (0.1)**	169 (18.8)	460 (38.1)
n = 10	5.9 (0.8)	14.8 (1.5)	61.1 (12.9)	82.4 (8.0)	415 (44.6)	21.7 (4.7)	13.6 (1.7)	46.3 (7.8)	42.8 (16.7)	176 (34.9)	2.1 (1.0)		
B34 -2	**0.6****	**1.5**	**8.9**	**5.9****	**45.0**	**4.1**	**2.0**	**3.8****	**7.3****	**20.5**	**0.4**	122	355*
	3.8	9.1	55.0	36.1	278	25.0	12.5	23.2	45.2	126	2.6		
B34 a	**0.6****	**1.5**	**5.2****	**6.9****	**56.1****	**1.8***	**1.4**	**4.0****	**2.3****	**20.3**	**0.0****	182	373
	5.4	14.5	50.0	66.6	541	17.5	13.2	38.4	22.1	196	0.0		
B34 b	**0.6****	**1.5**	**5.3****	**8.0**	**50.6****	**2.0***	**1.3**	**3.8****	**2.0****	**24.9**	**0.0****	208	283**
	6.1	16.8	58.3	88.8	561	21.7	14.6	42.4	22.1	276	0.0		
B34 f	**0.7**	**1.8**	**5.5***	**7.0****	**43.5**	**2.2**	**1.8**	**5.3**	**2.5****	**29.5****	**0.0****	158	497
	6.1	15.2	45.8	58.3	361	18.4	15.3	44.0	20.4	245	0.0		
B34 a5	**0.4****	**0.9****	**5.7***	**8.4**	**52.1****	**2.7**	**1.5**	**3.8****	**6.2**	**18.0**	**0.3**	183	221**
	3.8	8.4	55.0	80.5	499	25.8	14.6	36.0	59.4	173	2.6		
B34 c3	**0.8**	**1.6**	**10.1**	**9.8**	**47.4****	**3.2**	**1.7**	**4.6***	**6.3**	**14.2****	**0.2**	152	567**
	6.1	13.0	80.8	77.7	377	25.8	13.9	36.8	50.5	113	1.3		
B34 d3	**0.7***	**1.6**	**8.9**	**9.0**	**48.8****	**3.1**	**1.9**	**4.2****	**5.2**	**16.6***	**0.1***	170	570**
	6.1	14.5	80.0	80.5	438	27.5	16.6	37.6	47.0	149	1.3		
B34 d4	**0.7**	**1.4**	**10.7***	**9.2**	**46.0***	**3.1**	**1.4**	**4.4***	**5.8**	**17.2***	**0.1****	222	515
	8.4	16.7	125	108	538	35.8	16.7	52.0	67.3	201	1.3		
B34 Mean	**0.6 (0.1)**	**1.5 (0.3)**	**7.5 (2.3)**	**8.0 (1.3)**	**48.7 (4.1)**	**2.8 (0.8)**	**1.6 (0.3)**	**4.2 (0.5)**	**4.7 (2.1)**	**20.2 (5.0)**	**0.1 (0.2)**	175 (31.8)	423 (133)
n = 8	5.7 (1.5)	13.5 (3.2)	68.7 (26.2)	74.6 (21.5)	449 (103)	24.7 (5.8)	14.6 (1.5)	38.8 (8.2)	41.8 (18.2)	185 (56.3)	1.1 (1.1)		
	(μmol/ 25 ml 0.5% Driselase)												
Control	0.0 (0.0)	10.1 (15.9)	0.5 (1.3)	0.5 (1.1)	0.4 (0.9)	0.3 (0.9)	0.8 (1.7)	0.8 (2.3)	0.1 (0.3)	1.0 (1.3)	0.0 (0.0)	2.6 (3.3)	n.a.
n = 12													

Table 4.6 continued.

Line	Products of Driselase digestion (mol%; µmol/ g AIR)											Total mass of identified products (mg/ g AIR)	Mass of digestion residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	IP	X_2	GalA	GlcA		
Ws	**0.9 (0.1)**	**1.7 (0.2)**	**8.0 (1.0)**	**9.6 (1.0)**	**41.4 (2.0)**	**3.2 (0.5)**	**2.5 (0.6)**	**5.6 (0.4)**	**5.7 (0.6)**	**21.2 (1.7)**	**0.3 (0.1)**	168 (58.7)	441 (39.6)
n = 6	6.9 (0.6)	12.8 (1.2)	60.4 (7.5)	73.0 (9.1)	316 (48.5)	24.3 (5.1)	18.7 (3.4)	42.1 (4.1)	43.2 (6.8)	162 (31.5)	2.2 (0.5)		
Col	**0.7** (0.1)**	**1.7 (0.2)**	**6.9** (1.0)**	**9.4 (0.5)**	**47.1** (2.4)**	**2.5** (0.4)**	**1.5** (0.1)**	**5.3 (0.6)**	**4.8 (1.5)**	**20.0 (3.1)**	**0.3 (0.1)**	169 (18.8)	460 (38.1)
n = 10	5.9 (0.8)	14.8 (1.5)	61.1 (12.9)	82.4 (8.0)	415 (44.6)	21.7 (4.7)	13.6 (1.7)	46.3 (7.8)	42.8 (16.7)	176 (34.9)	2.1 (1.0)		
B52-1	**0.7***	**1.4**	**8.0**	**7.2***	**40.3**	**5.3****	**1.7**	**4.3****	**8.6****	**22.1**	**0.5****	248	219**
	8.4	17.5	102	91.6	516	67.5	22.2	55.2	111	283	6.4		
B52-3	**0.7***	**1.4**	**8.5**	**8.4**	**47.4****	**3.8**	**1.7**	**4.7**	**7.9****	**15.1****	**0.4***	171	403
	6.1	12.2	75.8	74.9	422	34.1	15.3	41.6	70.0	134	3.9		
B52-5	**0.5****	**1.4**	**7.3**	**6.5****	**42.9**	**4.7****	**1.9**	**4.4****	**8.1****	**21.7**	**0.6****	173	388
	4.6	12.2	65.0	58.3	383	41.7	17.3	39.2	72.6	193	5.2		
B52 a3	**0.8**	**1.6**	**11.3****	**10.8**	**40.7**	**2.6**	**1.5**	**5.3**	**5.4**	**19.8**	**0.3**	171	321**
	7.6	15.2	110	105	397	25.0	14.6	52.0	52.3	193	2.6		
B52 b3	**0.7**	**1.8**	**5.7***	**8.0**	**48.7****	**2.3**	**1.4**	**5.3**	**3.9****	**22.0**	**0.1****	229	265**
	8.4	21.3	66.6	94.3	572	27.5	16.0	62.4	46.1	258	1.3		
B52 d4	**0.5****	**1.3***	**6.1***	**8.3**	**48.1****	**2.9**	**1.5**	**3.9****	**5.7**	**21.5**	**0.3**	199	338*
	4.6	13.0	60.8	83.2	483	29.2	15.3	39.2	57.6	216	2.6		
B52 b	**1.6****	**5.0****	**8.0**	**4.6****	**31.3****	**3.9**	**2.6**	**12.4****	**8.1****	**22.4**	**0.0****	91.4	533*
	6.9	21.3	34.1	19.4	133	16.7	11.1	52.8	34.5	95.3	0.0		
B52 Mean	**0.8 (0.4)**	**2.0 (1.4)**	**7.8 (1.9)**	**7.7 (1.9)**	**42.8 (6.1)**	**3.6 (1.1)**	**1.8 (0.4)**	**5.8 (3.0)**	**6.8 (1.8)**	**20.7 (2.6)**	**0.3 (0.2)**	183 (50.6)	352 (102)
n = 7	6.6 (1.6)	16.1 (4.0)	73.5 (25.8)	75.3 (28.9)	415 (142)	34.5 (16.5)	15.9 (3.3)	48.9 (9.0)	63.4 (24.7)	196 (65.6)	3.1 (2.2)		
	(µmol/ 25 ml 0.5% Driselase)												
Control	0.0 (0.0)	10.1 (15.9)	0.5 (1.3)	0.5 (1.1)	0.4 (0.9)	0.3 (0.9)	0.8 (1.7)	0.8 (2.3)	0.1 (0.3)	1.0 (1.3)	0.0 (0.0)	2.6 (3.3)	n.a.
n = 12													

Table 4.6 continued.

Line	Products of Driselase digestion (mol%; µmol/ g AIR)											Total mass of identified products (mg/ g AIR)	Mass of digestion residue (mg/ g AIR)
	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	IP	X_2	GalA	GlcA		
Ws	**0.9 (0.1)**	**1.7 (0.2)**	**8.0 (1.0)**	**9.6 (1.0)**	**41.4 (2.0)**	**3.2 (0.5)**	**2.5 (0.6)**	**5.6 (0.4)**	**5.7 (0.6)**	**21.2 (1.7)**	**0.3 (0.1)**	168 (58.7)	441 (39.6)
n = 6	6.9 (0.6)	12.8 (1.2)	60.4 (7.5)	73.0 (9.1)	316 (48.5)	24.3 (5.1)	18.7 (3.4)	42.1 (4.1)	43.2 (6.8)	162 (31.5)	2.2 (0.5)		
Col	**0.7** (0.1)**	**1.7 (0.2)**	**6.9** (1.0)**	**9.4 (0.5)**	**47.1** (2.4)**	**2.5** (0.4)**	**1.5** (0.1)**	**5.3 (0.6)**	**4.8 (1.5)**	**20.0 (3.1)**	**0.3 (0.1)**	169 (18.8)	460 (38.1)
n = 10	5.9 (0.8)	14.8 (1.5)	61.1 (12.9)	82.4 (8.0)	415 (44.6)	21.7 (4.7)	13.6 (1.7)	46.3 (7.8)	42.8 (16.7)	176 (34.9)	2.1 (1.0)		
B58-1	**0.8**	**1.7**	**9.7**	**8.9**	**48.7****	**3.4**	**1.8**	**5.6**	**7.9****	**11.0****	**0.4***	163	347*
	6.9	15.2	85.8	79.2	432	30.5	15.9	49.6	70.0	97.9	3.9		
B58-6	**0.5****	**1.4**	**7.5**	**8.6**	**49.5****	**2.1**	**1.5**	**4.1****	**3.3****	**21.5**	**0.0****	153	549*
	4.6	12.2	63.3	73.1	420	17.5	12.5	35.2	28.3	183	0.0		
B58-9	**0.8**	**1.4**	**8.6**	**7.9**	**45.4***	**3.1**	**1.3**	**5.6**	**8.0****	**17.5***	**0.4**	115	436
	4.6	8.4	53.3	48.7	280	19.3	8.0	34.4	49.6	108	2.6		
B58 c2	**0.9**	**1.9**	**9.8**	**7.0****	**40.8**	**2.9**	**2.2**	**6.7****	**8.3****	**19.3**	**0.2**	131	310**
	6.1	13.0	68.3	48.7	283	20.3	15.3	46.4	57.6	134	1.3		
B58 d2	**0.6****	**1.5**	**8.5**	**8.0**	**43.1**	**3.3**	**1.7**	**5.2**	**8.0****	**20.2**	**0.0****	176	271**
	6.1	13.7	80.0	76.1	408	30.8	15.8	49.6	75.3	191	0.0		
B58 Mean	**0.7 (0.1)**	**1.6 (0.2)**	**8.8 (1.0)**	**8.1 (0.7)**	**45.5 (3.7)**	**3.0 (0.5)**	**1.7 (0.3)**	**5.5 (0.9)**	**7.1 (2.1)**	**17.9 (4.1)**	**0.2 (0.2)**	147 (24.7)	383 (111)
n = 5	5.7 (1.0)	12.5 (2.6)	70.1 (13.0)	65.2 (15.2)	365 (76.4)	23.7 (6.5)	13.5 (3.4)	43.0 (7.6)	56.2 (18.6)	143 (42.4)	1.6 (1.7)		
	(µmol/ 25 ml 0.5% Driselase)												
Control	0.0 (0.0)	10.1 (15.9)	0.5 (1.3)	0.5 (1.1)	0.4 (0.9)	0.3 (0.9)	0.8 (1.7)	0.8 (2.3)	0.1 (0.3)	1.0 (1.3)	0.0 (0.0)	2.6 (3.3)	n.a.
n = 12													

4.3.3.4 Unidentified carbohydrate digestion products

A number of products of the Driselase digestion were not identified (Peaks 1–3, Figure 4.8b). The areas of these peaks as a percentage of the total chromatogram area are shown in Table 4.7. The corresponding values for xylobiose are shown for comparison.

There were a number of significant differences between Ws and the other *Arabidopsis* lines. Peak 1 was significantly smaller than in Ws in two individuals from each of B16 and B17, and in one individual from each of B14 and B24. The proportion of the chromatogram area that was peak 2 was significantly different from Ws for individuals from seven Feldmann lines (B14, B16, B17, B24, B27, B34 and B52). However, peaks 1 and 2 were minor components of the Ws digest, being only 2.5% and 2.2% of the total chromatogram area.

For Columbia and individuals from all the Feldmann lines, peak 3 made up a significantly smaller proportion of the chromatogram than for Ws (Table 4.7). For the Feldmann lines peak 3 ranged from 0% (B17 c2, B24 b, B34-2, B52-1, B52 b3, B58-1 and B58-9) to 72% (B24 j) of the Ws value. Peak 3 is actually composed of a number of smaller peaks (Figure 4.8c). For all of the Feldmann lines at least 86% of the individuals analysed showed a significant difference from Ws in the relative size of peak 3.

Glucuronic acid is present in the AIR linked by α- and β- bonds in xylan and the rhamnogalacturonans, respectively. The glucuronic acid present in xylan would not be present in the Driselase products as the monosaccharide as there is no α-glucuronidase activity in Driselase (see Section 3.1.3.4). Instead the glucuronic acid from xylan would be present as (methyl)glucuronosyl-xylose or (methyl)glucuronosyl-xylosyl-xylose. It is possible that the unidentified peaks in the HPLC chromatograms were due to these di- or trisaccharides.

Table 4.7 The unidentified Driselase digestion products of *A. thaliana* AIR. The values shown in bold are means with standard deviations in brackets; n = number of replicate digests of different preparations of AIR; ** = probability of less than 0.05 that the values come from populations with the same mean (as calculated by the anova method [comparisons between Ws and Columbia] and the Student's t distribution [comparisons between Ws and the Feldmann lines], Section 2.2.6). The values for xylobiose are shown for comparison (statistical analysis was not carried out for the X_2 figures).

	Percentage of total chromatogram area			
Line	**Peak 1**	**Peak 2**	**Peak 3**	**X_2**
Ws	**2.5**	**2.2**	**9.7**	**5.5**
n = 6	(0.8)	(0.1)	(1.5)	(0.5)
Col	**3.1**	**2.0**	**4.3 ****	**6.5**
n = 10	(0.5)	(0.3)	(4.9)	(1.7)
B5-2	0.7	2.2	1.0**	4.1
B5-3'	1.2	2.3	1.3**	8.6
B5 a5	1.6	2.1	1.4**	0.8
B5 a6	1.6	1.9	2.7**	7.3
B5 b4	1.5	2.1	1.8**	10.2
B5 c4	1.6	2.0	1.4**	10.0
B5 Mean	**1.4**	**2.1**	**1.6**	**6.8**
n = 6	(0.4)	(0.1)	(0.6)	(3.7)
B14-1	1.8	1.8**	2.5**	4.6
B14-2	1.5	2.2	2.0**	9.7
B14-3	1.5	2.1	2.8**	9.7
B14-5	1.5	2.3	1.7**	9.6
B14-6	1.3	2.2	2.5**	4.9
B14 c	1.6	2.1	1.1**	12.2
B14 g	1.3	1.9	2.4**	6.4
B14 h	0.0 **	3.4**	2.4**	5.5
B14 j	1.1	1.7**	1.7**	3.8
B14 Mean	**1.3**	**2.2**	**2.1**	**7.4**
n = 9	(0.5)	(0.5)	(0.5)	(3.0)
B16-2	1.2	1.8**	1.3**	9.8
B16-4	1.7	2.3	1.7**	13.4
B16 a1	0.0**	1.7**	1.8**	10.3
B16 b1	1.6	1.7**	3.0**	6.9
B16 c	0.4**	2.2	2.1**	3.6
B16 i	1.2	1.9	2.7**	4.0
B16 Mean	**1.0**	**1.9**	**2.1**	**8.0**
n = 6	(0.7)	(0.3)	(0.6)	(3.8)

Table 4.7 cont.

Line	Percentage of total chromatogram area			
	Peak 1	Peak 2	Peak 3	X_2
Ws	**2.5**	**2.2**	**9.7**	**5.5**
n = 6	(0.8)	(0.1)	(1.5)	(0.5)
Col	**3.1**	**2.0**	**4.3 ****	**6.5**
n = 10	(0.5)	(0.3)	(4.9)	(1.7)
B17 b1	1.8	2.1	1.3**	9.0
B17 b2	2.1	2.2	3.5**	8.0
B17 c1	0.0**	0.6**	0.1**	11.2
B17 c2	0.0**	1.3**	0.0**	7.9
B17 d2	3.2	1.8**	1.8**	8.4
B17 Mean	**1.4**	**1.6**	**1.4**	**8.9**
n = 5	(1.4)	(0.7)	(1.4)	(1.4)
B24 b	2.4	2.0	0.0**	4.5
B24 c	2.1	2.0	1.9**	4.3
B24 f	1.8	1.6**	1.7**	3.9
B24 g	1.5	2.1	0.9**	4.6
B24 h	1.4	2.2	1.0**	4.2
B24 j	0.0**	1.2**	6.8	5.4
B24-4	3.0	2.2	1.7**	8.2
B24 Mean	**1.8**	**1.9**	**2.0**	**5.0**
n = 7	(0.9)	(0.4)	(2.2)	(1.5)
B27-6	2.5	2.1	1.1**	9.0
B27 b	2.2	2.2	2.8**	4.1
B27 f	3.3	1.9	2.2**	4.1
B27 g	2.5	1.7**	2.6**	3.3
B27 h	2.6	1.8**	3.9**	3.7
B27 Mean	**2.6**	**1.9**	**2.5**	**4.8**
n = 5	(0.4)	(0.2)	(1.0)	(2.3)
B34-2	2.1	3.0**	0.0**	12.8
B34 a	2.2	2.2	3.4**	10.2
B34 b	2.1	1.7**	1.8**	9.2
B34 f	2.3	1.9	3.4**	8.7
B34 a5	3.2	1.5**	2.1**	9.4
B34 c3	1.9	2.0	5.2	5.0
B34 d3	2.4	1.6**	3.5**	4.2
B34 d4	2.1	2.2	2.4**	4.4
B34 Mean	**2.3**	**2.0**	**2.7**	**8.0**
n = 8	(0.4)	(0.5)	(1.5)	(3.1)

Table 4.7 cont.

	Percentage of total chromatogram area			
Line	**Peak 1**	**Peak 2**	**Peak 3**	X_2
Ws	**2.5**	**2.2**	**9.7**	**5.5**
n = 6	(0.8)	(0.1)	(1.5)	(0.5)
Col	**3.1**	**2.0**	**4.3 ****	**6.5**
n = 10	(0.5)	(0.3)	(4.9)	(1.7)
B52-1	2.6	2.7**	0.0**	12.9
B52-3	1.4	2.5	5.8	10.6
B52-5	2.5	2.5	4.0**	11.1
B52 a3	1.8	2.5	1.0**	6.3
B52 b3	3.1	1.9	0.0**	6.5
B52 d4	1.8	2.2	3.3**	6.7
B52 b	2.3	2.6**	1.0**	9.6
B52 Mean	**2.2**	**2.4**	**2.1**	**9.1**
n = 7	(0.6)	(0.3)	(2.2)	(2.6)
B58-1	2.1	2.5	0.0**	9.5
B58-6	1.6	2.0	5.1**	5.2
B58-9	1.6	2.5	0.0**	9.7
B58 c2	1.8	2.4	3.0**	8.3
B58 d2	1.6	2.5	3.3**	10.8
B58 Mean	**1.7**	**2.4**	**2.3**	**8.7**
n = 5	(0.2)	(0.2)	(2.2)	(2.1)

4.3.4 Discussion

Tables 4.5 and 4.6 give the proportions (mol%) and the yields (μmol/ g AIR) of the screen products. The use of proportions removes variability due to differences in the proportion of the AIR solubilised and allows comparisons to be made between *A. thaliana* lines or individual plants. The yields of the products allow comparisons between the results of the two screens for a particular *A. thaliana* line.

Wassilewskija (Ws) and Columbia are genetically homogeneous lines so statistically they were treated as a group and comparisons made between the groups by the analysis of variance (anova, Section 2.2.6).

The nine Feldmann lines identified by the repeat screen are all from the T4 generation (Section 2.2.1.2, Feldmann and Marks, 1987). The T2 generation was selected by kanamycin resistance; therefore the T4 population will be 75% kanamycin resistant plants and 25% kanamycin sensitive plants (Section 2.2.1.2, Figure 2.1). As each population of Feldmann lines was genetically heterogeneous they cannot be treated as a group statistically. Individual plants from each of the Feldmann lines were compared to the group of Ws plants using Student's t distribution (Section 2.2.6).

For a difference between Ws and a Feldmann line in one of the screen products to be considered due to the presence of the T-DNA tag in the Feldmann line, the percentage of the group with the significant difference from Ws, should (theoretically) be 75% (if the proposed mutation was dominant) or 58% (if the proposed mutation was recessive). In the following analysis a significant difference from Ws in approximately 75% or more of the population was taken to indicate that the difference was due to a dominant mutation caused by the presence of the T-DNA tag in the individuals concerned. If between approximately 58-75% of the population had the significant difference from Ws the difference could be due to a recessive mutation caused by the presence of the T-DNA tag. A summary of

the statistically significant differences from Ws which may be due to the presence of a T-DNA tag in the *A. thaliana* lines is given in Table 4.11 (at the end of Section 4.3.4.12).

4.3.4.1 Screen products for *A. thaliana* ecotype Wassilewskija

Table 4.8 gives the yields (μmol/ g AIR) of the screen products for Wassilewskija (Ws). This allows comparison between the results of the two screens for Ws.

Residue mass and mass of total identified products

The mass of the residue remaining after TFA hydrolysis of Ws AIR was significantly smaller than that of Driselase digestion by 21% (Table 4.8). This was surprising as TFA hydrolysis does not break down cellulose while Driselase digestion does. The difference in residue mass was approximately 9% of the AIR mass.

There was no significant difference between the total mass of identified products for the two screens (Table 4.8). This indicated that the identified products of the two screens made up approximately the same proportion of the AIR (approximately 17-18% of the AIR mass). Again, this was surprising as TFA hydrolysis does not break down the majority of cellulose (Selvendran and Ryden, 1990) while Driselase digestion does. However, there were a number of unidentified products of Driselase digestion that may account for this (Section 4.3.3.4).

Since there was no difference in the total mass of identified products for the two screens, the difference in residue mass may be due to the solubilisation by TFA of a non-carbohydrate component, the hydrolysis products of which were not detected by the HPLC system used.

The AIR is composed of the cell wall polysaccharides, starch, protoplasmic proteins and RNA. It was unlikely that the difference in residue mass was due to increased solubilisation of proteins by TFA as these tend to be relatively acid-stabile, although it is possible that some

oligopeptides were produced. The concentration of RNA in the AIR had not been determined so a judgement can not be made on whether its increased solubilisation could be responsible for the observed decrease in residue mass. However, a ribose marker was run on the TLCs for the initial and repeat-screens (Figures 4.1 and 4.4) and a stained ribose spot was not observed for Columbia or any of the screened lines. Therefore, if ribose is present in the TFA products it is at a lower concentration than arabinose (11.7 mg/ g AIR for Ws).

Glucose

The yield of glucose in the Driselase products was significantly (by 209%) higher than in the TFA products. The figures for glucose in Table 4.8 include the glucose present in isoprimeverose. The glucose in the TFA hydrolysate is primarily a product of xyloglucan and starch with a minor amount derived from rhamnogalacturonan II. In addition, Driselase also breaks down cellulose into glucose but Driselase does not efficiently convert starch to glucose.

The yield of cellulose-derived glucose in the Driselase products can be approximated by calculating the yield of glucose that would be due to xyloglucan (Table 4.9). The yield of cellulose-derived glucose in the Driselase digest was 302 μmol glucose/ g AIR, which implies that Driselase-susceptible cellulose makes up approximately 4.9% of the AIR by mass. The yield of starch-derived glucose can be calculated using the total yield of glucose which would be due to xyloglucan (Xyl_{XG}, Table 4.9), and was found to be 65.1 μmol/ g AIR, which implies that approximately 1.1% of the AIR, by mass, was starch.

Xylose

The yield of total xylose in the Driselase products was significantly lower than in the TFA products by 66%. The figure for xylose given in Table

4.8 includes the xylose present in xylobiose and isoprimeverose. The significant difference in total xylose indicates that more xylose-containing polysaccharides were broken down to identified products by TFA hydrolysis than by Driselase digestion. The degree of substitution (with [methyl]glucuronic acid, arabinose or acetyl groups) of the xylose residues in xylan would affect the proportion of xylose detected in the digest as these xylose residues would be more resistant to Driselase digestion than unsubstituted residues (Section 3.1.3.4; Fry, 1988).

The yields of isoprimeverose and xylobiose in the digest were considered to be indicative of, respectively, the proportions of xyloglucan and xylan in the AIR. These figures give a mass ratio (mass of isoprimeverose/ mass of xylobiose) of approximately 1. The yields of polysaccharides found by Zablackis et al (1995) in *A. thaliana* leaves give a ratio of xyloglucan to xylan of approximately 5. However, there will be a larger proportion of xylan in bolting stems due to secondary growth in xylem and phloem tissues.

Assuming that all the xylan and xyloglucan present in the AIR was hydrolysed by TFA, the difference between the yield of xylose in the TFA and Driselase products gives the yield of Driselase-resistant xylose present in the AIR (Table 4.10). For Ws, this Driselase-resistant xylose accounts for 291 μmol/ g AIR. About 66% of the total xylose was Driselase-resistant in Ws (Table 4.10).

The majority of the Driselase-susceptible xylose from the backbone of xylan was present in the digest products as xylobiose. The free xylose in the digest is primarily derived from the side chains of xylan, with a small amount due to rhamnogalacturonans. The proportion of free xylose in the digestion products of Ws was 56% of the proportion of xylobiose.

Galacturonic acid and rhamnose

The yields of galacturonic acid in the products of the two screens were not significantly different (Table 4.8). Galacturonic acid glycosyl bonds

are relatively resistant to the acid hydrolysis conditions used and the liberated galacturonic acid is more susceptible to degradation (e.g. decarboxylation) in the hot acid than are most neutral sugars. This would indicate that the observed yield of galacturonic acid in the TFA hydrolysate (and also, therefore, in the Driselase products) was lower than the yield of galacturonic acid in the AIR.

The yield of rhamnose in the Driselase products was significantly lower than in the TFA products by 82% (Table 4.8). The differences in rhamnose yield imply that TFA hydrolysis breaks down a significantly larger proportion of the pectins than digestion with Driselase. This could indicate that a proportion of the rhamnose is Driselase-resistant, possibly due to acetylation. The increase in rhamnose in the TFA hydrolysate was approximately 60 μmol/ g AIR, while the yield of galacturonic acid was approximately the same in both screens.

Galactose

The yield of galactose in the Driselase products was significantly (36%) lower than in the TFA products (Table 4.8). TFA hydrolysis solubilised 41.5 μmol/ g AIR more galactose than Driselase digestion. This difference in galactose yield could be due to differences in susceptibility of xyloglucan or the rhamnogalacturonans to the two screening methods.

Arabinose

The yield of arabinose in the Driselase products was 22% lower than in the TFA products (Table 4.8). This could indicate a reduced susceptibility of xylans, rhamnogalacturonans or glycoproteins to Driselase digestion compared to TFA hydrolysis.

Mannose

TFA solubilised 3.7 times more mannose than Driselase digestion (Table 4.8). β-Mannose could be present in the AIR of *A. thaliana* in glucomannans and α-mannose in glycoproteins. Both α-mannosidase and β-mannosidase activities are present in Driselase (Fry, 1988). This result could again indicate a greater susceptibility of polysaccharides in the AIR to acid hydrolysis than to enzymatic digestion.

Fucose

The yield of fucose in the Driselase products was significantly (23%) less than in the TFA products. Fucose is only a minor component of both screens (Tables 4.5 and 4.6) and the difference between the yields of fucose in the screen products was only 2 μmol/ g AIR. However, the data could indicate a greater susceptibility of fucose in xyloglucan or the rhamnogalacturonans to TFA.

Glucuronic acid

TFA hydrolysis solubilised seven times more glucuronic acid than Driselase digestion, despite uronic acid bonds being relatively resistant to the acid hydrolysis conditions used. Glucuronic acid is present in the AIR in the side chains of xylan and rhamnogalacturonan-II. Glucuronic acid in xylan is linked by α-(1→2) and α-(1→3) bonds to the xylose backbone, while in rhamnogalacturonan-II it is linked by β-(1→4) bonds to fucose within the side chains. The glucuronic acid present in xylan would not be present in the Driselase products as the monosaccharide because there is no α-glucuronidase activity; therefore, the glucuronic acid in the Driselase products will be derived from the pectins.

Summary

The AIR of Ws was more susceptible to TFA hydrolysis than Driselase digestion, but there was no significant difference between the total mass of

identified products of both screens, despite the fact that Driselase breaks down cellulose while TFA does not. There were some unidentified products of Driselase digestion which were not taken into account in the total mass of identified products.

Cellulose is broken down by Driselase digestion and not TFA hydrolysis. Starch is hydrolysed by TFA hydrolysis while Driselase does not efficiently convert starch to glucose. The xylose, galacturonic acid, rhamnose, galactose, arabinose, mannose, fucose and glucuronic acid in the AIR were all more susceptible to TFA hydrolysis than Driselase digestion. This indicates that a percentage of the polysaccharides containing these monosaccharides are Driselase-resistant. Therefore, a percentage of the following cell wall constituents may be Driselase-resistant: the pectic polysaccharides, xylan, xyloglucan, mannans, and the carbohydrate component of glycoproteins.

Table 4.8 Comparison of the screen products derived from TFA hydrolysis and Driselase digestion of the AIR of *A. thaliana* ecotype Wassilewskija (Ws). The AIR was extracted as described in Section 2.3.3.3, and either hydrolysed with TFA (Section 2.3.4.2) or incubated with Driselase (Section 2.3.4.1). Screen products were separated on a Dionex HPLC (Section 2.3.5.3). The values given are means with standard deviations in brackets. n = number of replicates; 1 = these values are the total amount of the sugar found in the Driselase products (the glucose value includes the glucose found in the digest in the form of isoprimeverose [42.1 µmol Glc/ g AIR] and the xylose figure includes xylose present in the digest in the form of isoprimeverose [42.1 µmol Xyl/ g AIR] and xylobiose [86.4 µmol Xyl/ g AIR]); P = probability that the values come from populations with the same mean (as calculated by the anova method, Section 2.2.6).

	Screen products (µmol / g AIR)									Total identified products	Residue
Screen	**Fuc**	**Rha**	**Ara**	**Gal**	**Glc**	**Xyl**	**Man**	**GalA**	**GlcA**	**(mg/g AIR)**	**(mg/g AIR)**
Driselase digestion n = 6	6.9 (0.6)	12.8 (1.2)	60.4 (7.5)	73.0 (9.1)	375 [1] (32.7)	153 [1] (21.1)	18.7 (3.4)	162 (31.5)	2.2 (0.5)	168 (58.7)	441 (39.6)
TFA hydrolysis n = 6	8.9 (1.1)	72.6 (8.6)	77.8 (11.5)	114 (14.4)	121 (19.7)	444 (103)	68.3 (17.6)	167 (23.8)	15.5 (4.9)	182 (28.2)	348 (52.0)
P =	< 0.005	< 0.001	< 0.025	< 0.001	< 0.001	< 0.001	< 0.001	> 0.75	< 0.001	> 0.50	< 0.01

Table 4.9 The calculated yields of glucose derived from cellulose, starch and xyloglucan. **1** = yield of glucose in the Driselase digest; **2** = yield of isoprimeverose in the Driselase digest; **3** = yield of glucose in the TFA hydrolysate; **4** = glucose derived from cellulose (= $Glc_D - \frac{1}{3}Glc_{IP}$]); **5** = glucose derived from xyloglucan (= $\frac{4}{3}Glc_{IP}$); **6** = glucose derived primarily from starch (= Glc_T - Glc_{XG}). Figures in bold are percentages of the Ws values.

Line	Glc_D [1] (µmol/ g AIR)	Glc_{IP} [2] (µmol/ g AIR)	Glc_T [3] (µmol/ g AIR)	Glc_C [4] (µmol/ g AIR)	Glc_{XG} [5] (µmol/ g AIR)	Glc_S [6] (µmol/ g AIR)
Ws	316	42.1	121	302	56.1	65.1
				100	**100**	**100**
Col	428	50.7	149	411	67.6	81.5
				136	**120**	**125**

Table 4.10 Driselase-resistance of xylose in *Arabidopsis* AIR. **1** = yield of xylose in TFA hydrolysate; **2** = total yield of xylose in the Driselase digest (including xylose present in the form of xylobiose and isoprimeverose); **3** = Driselase-resistant xylose (= Xyl_T – Xyl_D). Figures in bold are percentages of the Ws values.

Line	Xyl_T [1] (µmol/ g AIR)	Xyl_D [2] (µmol/ g AIR)	Xyl_{DR} [3] (µmol/ g AIR)	Xyl_{DR} as a % of Xyl_T
Ws	444	153	291	65.6
			100	**100**
Col	466	182	284	60.9
			97.5	**92.8**

4.3.4.2 Screen products for *A. thaliana* ecotype Columbia

Residue mass and mass of total identified products

For both TFA hydrolysis and Driselase digestion the mass of the residues and the total mass of identified products were not significantly different from Ws (Tables 4.5 and 4.6).

Glucose

The proportion of glucose was 42% higher than in Ws in the TFA products and 14% higher in the Driselase products (Tables 4.5 and 4.6). As the increase was seen in both screens it was not only due to cellulose. The starch-, xyloglucan- and cellulose-derived glucose were 25%, 20% and 36% higher, respectively, than in Ws (Table 4.9). A very small proportion of the glucose in the screen products is probably derived from the side chains of rhamnogalacturonan II. This can be assumed to be negligible when compared to the yield of glucose derived from cellulose, xyloglucan and starch. Therefore the increase in the proportion of glucose in the two screens was due to an increase, when compared to Ws, in the proportions of starch and cellulose found in the AIR of Columbia.

Xylose

The proportions of isoprimeverose and xylobiose in the Driselase screen were approximately the same as in Ws; while the proportion of xylose in the Driselase screen was 23% lower (a difference of only 0.7 mol%) than in Ws and approximately equal to that of Ws in the TFA screen (Tables 4.5 and 4.6). The proportion of free xylose in the digestion products was 52% of the proportion of xylobiose, which is approximately the same as in Ws. The percentage of the total xylose that was Driselase-resistant was also found to be approximately that of Ws (Table 4.10).

Galacturonic acid and rhamnose

The rhamnose and galacturonic acid proportions were not significantly different from those found in Ws for either the TFA or the Driselase screens, indicating that the proportions of pectins susceptible to the screens were not different from those found in Ws (Tables 4.5 and 4.6).

Galactose

The proportion of galactose in the TFA products was significantly higher than in Ws, while it was approximately the same as in Ws in the Driselase screen (Tables 4.5 and 4.6). The difference between the yields of galactose in the TFA hydrolysates of Columbia and Ws was very small and considered to be negligible.

Arabinose

The proportion of arabinose was 14% lower in Columbia than in Ws in the Driselase products and was approximately that of Ws in the TFA products (Tables 4.5 and 4.6). The difference between Ws and Columbia in the yield of arabinose in the Driselase products was very small (0.7 μmol/ g AIR) and considered to be negligible. Arabinose is present mainly in the side chains of rhamnogalacturonans and xylan and also the carbohydrate component of glycoproteins.

Mannose

The mannose proportion was significantly lower in both screens, than in Ws. The difference between the Ws and Columbia yields of mannose in the TFA screen was 16.8 μmol/ g AIR, while for the Driselase screen the difference was 5.1 μmol/ g AIR (Tables 4.5 and 4.6). These small changes could be due to a decreased proportion of mannose in the glycoproteins or of glucomannans.

Fucose

Fucose was significantly lower (by 25%) than in Ws in the Driselase products and was approximately the same as in Ws in the TFA products (Tables 4.5 and 4.6). The difference between Ws and Columbia in the yield of fucose in the Driselase products was approximately 1.0 μmol/ g AIR. This is a very small difference and can be considered to be negligible. Fucose is found in the side chains of xyloglucan, rhamnogalacturonans and glycoproteins.

Glucuronic acid

The proportion of glucuronic acid in the Driselase products was the same as for Ws, while the proportion in the TFA products was insignificantly lower (by 29%) than in Ws (Tables 4.5 and 4.6). This indicates that there were no significant differences between Ws and Columbia in the TFA- and Driselase-susceptibility of glucuronic acid.

Summary

The AIR of Columbia showed the same susceptibility to the two screens as that of Ws, in terms of the mass solubilised and the total mass of identified carbohydrate products.

The yields of starch-, xyloglucan- and cellulose-derived glucose were all higher (25%, 20% and 36%, respectively) than in Ws. The yield of total xylose was approximately the same in Columbia as in Ws, and the percentage of total xylose that was Driselase-resistant was only slightly lower than in Ws. There were no significant differences between Ws and Columbia in the susceptibility of glucuronic acid to the two screens.

The proportions of pectins susceptible to the screens were not different from those found in Ws. The difference between the yields of galactose, arabinose and fucose in the screen products of Columbia and Ws were all very small and considered to be negligible. There was less TFA-

and Driselase-susceptible mannose in Columbia than in Ws. This could be due to a decreased proportion of mannose in the glycoproteins or of glucomannans.

4.3.4.3 Screen products for the *A. thaliana* line B5

Residue mass and mass of total identified products

The total mass of identified products of TFA hydrolysis was significantly different from Ws in only 14% of B5 individuals, while for the total mass of identified products of Driselase digestion there were no significant differences between Ws and any of the B5 individuals (Tables 4.5 and 4.6). The mass of the TFA and Driselase residues were significantly different from Ws in 14% and 50% of B5 individuals, respectively. Although for the Driselase residue, 33% had lighter residues than Ws and 17% heavier. These results indicate that there were no consistent differences between the T-DNA tagged B5 population and Ws in the susceptibility of the AIRs to the two screens.

Glucose

The proportions of glucose in the TFA and Driselase products were significantly different from Ws in only 14% and 33% of B5 individuals, respectively. All of the B5 individuals had a significantly higher proportion of isoprimeverose (from 16-43% more than Ws) present in the Driselase products than Ws. These results indicate that there were no consistent differences between Ws and the T-DNA tagged B5 population in the amount of cellulose-derived glucose and also the total amount of non-cellulosic-derived glucose. However, the results also indicate that there was more Driselase-susceptible xyloglucan in the T-DNA tagged B5 population than in Ws. It is possible that the level of another non-cellulosic glucose-containing polysaccharide (e.g. starch) is lower than in Ws, compensating for the higher level of glucose derived from xyloglucan.

Xylose

The proportion of xylobiose in the Driselase screen was significantly higher than Ws (from 72 to 132% more than Ws) in 83% of the B5 individuals. This indicated that the proportion of Driselase-susceptible xylan was higher in the majority of B5 individuals than in Ws (Table 4.6). As stated above all of the B5 individuals had higher levels of Driselase-susceptible xyloglucan than Ws. Higher levels of xyloglucan and xylan in the B5 AIRs would be expected to give higher proportions of xylose in the TFA products. However, only 14% of the B5 individuals had a proportion of xylose significantly lower than Ws (Table 4.5). This could indicate that the total amount of xylose in the AIR was not different from the wild type but its susceptibility to Driselase had changed.

There were no consistent significant differences between Ws and the B5 lines in the proportion of free xylose present in the Driselase products.

Galacturonic acid and rhamnose

The proportion of galacturonic acid in the TFA screen was significantly different than Ws in 43% of the B5 individuals. In the Driselase screen 83% of individuals had significantly less galacturonic acid than Ws (by 23-42%). The TFA products of 57% of B5 individuals had a significantly lower (by 27-31%) proportion of rhamnose than Ws (Table 4.5). The proportion of rhamnose in the Driselase products differed significantly from Ws in 33% of individuals, but half had higher levels than Ws and half lower levels. These results indicate that there was less Driselase-susceptible galacturonic acid and (possibly) less TFA-susceptible rhamnose in the T-DNA tagged B5 population than in Ws.

Galactose

For the TFA screen only 14% of the B5 individuals had significantly more galactose than Ws and in the Driselase screen 33% of individuals had significantly less galactose than Ws (Tables 4.5 and 4.6). These results

indicate that there were no consistent differences between the T-DNA tagged B5 population and Ws in the susceptibility of galactose to the two screens.

Arabinose

There was significantly more arabinose (by 33-51%) in the Driselase products of 83% of B5 individuals than in Ws (Table 4.6), while there were significant differences between Ws and B5 in the TFA products of only 29% of individuals (Table 4.5). This would appear to indicate that there was more Driselase-susceptible arabinose in the T-DNA tagged B5 population than in Ws.

Mannose

There were no significant differences between any of the B5 individuals and Ws in the proportion of mannose present in the Driselase products. In the TFA screen only 14% of individuals had a significantly different proportion of mannose from Ws (Tables 4.5 and 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B5 population and Ws in the susceptibility of mannose to the two screens.

Fucose

The proportion of fucose in the TFA products was significantly different from Ws for only 14% of B5 individuals. In the Driselase screen 33% of individuals had significantly less fucose than Ws (Table 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B5 population and Ws in the susceptibility of fucose to the two screens.

Glucuronic acid

The proportion of glucuronic acid in the TFA products was significantly lower than in Ws in 43% of B5 individuals, and in the Driselase products the proportion was significantly higher than in Ws in 50% of

individuals (Tables 4.5 and 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B5 population and Ws in the susceptibility of glucuronic acid to the two screens.

Summary

The results indicate that the presence of the T-DNA tag in the B5 population had no effect on the proportion of the AIRs that were susceptible to the two screens

The presence of the T-DNA tag in the B5 population had no effect on the amount of cellulose-derived glucose in the AIRs, **but did produce a higher level of Driselase-susceptible xyloglucan and xylan than in Ws. The difference between Ws and B5 in the Driselase-susceptibility of xylan could be due to a lower level of substituted xylose residues (with [methyl]glucuronic acid residues, arabinose residues and acetyl groups) in B5 than Ws.** The level of another non-cellulosic polysaccharide (e.g. starch) may be lower in the T-DNA tagged B5 population than Ws.

The presence of the T-DNA tag in the B5 population appears to have produced less Driselase-susceptible galacturonic acid and more Driselase-susceptible arabinose than in Ws. The T-DNA tagged B5 population could also have less TFA-susceptible rhamnose, due to a recessive mutation (as the percentage of the population showing the significant difference was 57%). These results could be due to changes in the structure and/ or the substitution pattern of the pectic polysaccharides (galacturonic acid and rhamnose), xylan (arabinose), arabinogalactans (arabinose) or the oligosaccharide side-chains of glycoproteins (arabinose).

4.3.4.4 Screen products for the *A. thaliana* line B14

Residue mass and mass of total identified products

In the TFA screen 29% and 14% of B14 individuals had a significant difference from Ws in the mass of the residues and the total mass of identified products, respectively (Table 4.5). For the Driselase screen, all B14 individuals had approximately the same total mass of identified products as Ws, and 44% of individuals had significantly different residue masses from Ws, although 33% had heavier residues and 11% had lighter residues (Table 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B5 population and Ws in the susceptibility of the AIRs to the two screens.

Glucose

Only 22% of B14 individuals had significantly more glucose in the Driselase products than Ws, but all the B14 individuals had significantly more isoprimeverose (by 20-100%) than Ws (Table 4.6). This indicates a higher level of xyloglucan in the T-DNA tagged B14 population than Ws. If this were true the expected result would be more glucose in the TFA products. However, only 14% of individuals had significantly more TFA-susceptible glucose than Ws. This indicates that there was no overall difference between the T-DNA tagged B14 population and Ws in the level of non-cellulosic-derived glucose in B14 than Ws. It is possible that the level of another glucose-containing non-cellulosic polysaccharide (e.g. starch) was lower in the T-DNA tagged B14 population than in Ws. This would explain why a higher level of non-cellulosic-derived glucose was not observed even though there was more xyloglucan-derived glucose.

Xylose

The proportion of the TFA products that was xylose was not significantly different from Ws for any of the B14 individuals (Table 4.5), indicating that the total amount of xylose in the T-DNA tagged B14

population was approximately the same as in Ws. The proportion of xylobiose was significantly different from Ws in 55% of individuals but 44% had more xylobiose than Ws and 11% had less (Table 4.6). Therefore, there were no consistent differences between the T-DNA tagged B14 population and Ws in the level of Driselase-susceptible xylan.

As stated above, the proportion of the Driselase products that was isoprimeverose was significantly higher than in Ws for all the individuals screened. The proportion of xylose in the Driselase products was significantly lower than in Ws in only 44% of individuals.

Galacturonic acid and rhamnose

In the Driselase products of B14 the proportion of rhamnose was significantly different from Ws in only 11% of individuals. The proportion of galacturonic acid in the Driselase screen was significantly different from Ws in 54% of individuals, but 44% had lower levels and 11% higher levels than Ws. In the TFA products rhamnose was significantly lower in 29% of individuals; while galacturonic acid was significantly higher in 14% of individuals and significantly lower in 29%, compared to Ws (Table 4.5). These results indicate that there were no consistent differences between the T-DNA tagged B14 population and Ws in the susceptibility of galacturonic acid or rhamnose to the two screens.

Galactose

The proportions of galactose in the TFA and Driselase products were significantly different than Ws in 29% and 33% of B14 individuals, respectively (Tables 4.5 and 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B14 population and Ws in the susceptibility of galactose to the two screens.

Arabinose

The proportion of arabinose in the TFA products was significantly higher than in Ws in 43% of B14 individuals (Table 4.5). In the Driselase products the proportion was only significantly different from Ws in 11% individuals (Table 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B14 population and Ws in the susceptibility arabinose to the two screens.

Mannose

The proportion of mannose in the TFA products was significantly different from Ws in only 14% of B14 individuals screened (Table 4.5), and in the Driselase products the proportion was significantly lower than in Ws in only 11% of individuals (Table 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B14 population and Ws in the susceptibility of mannose to the two screens.

Fucose

The proportion of fucose in the TFA products of B14 was significantly different from Ws in only 14% of individuals; while in the Driselase products the proportion of fucose was significantly lower (by 22-33%) than in Ws in 77% of individuals (Tables 4.5 and 4.6). This would appear to indicate that there was less Driselase-susceptible fucose in the T-DNA tagged B14 population than in Ws.

Glucuronic acid

The proportion of glucuronic acid in the Driselase products was significantly less than Ws in 33% of B14 individuals and more than in Ws in 11% of individuals. In the TFA products the proportion was significantly lower than Ws for 43% of individuals (Table 4.5). These results indicate that there were no consistent differences between the T-DNA tagged B14 population and Ws in the susceptibility of glucuronic acid to the two screens.

Summary

The results indicate that the presence of the T-DNA tag in the B14 population had no effect on the proportion of the AIRs that were susceptible to the two screens

The presence of the T-DNA tag in the B14 population had no effect on the amount of cellulose-derived glucose in the AIRs, **but did produce a higher level of Driselase-susceptible xyloglucan than in Ws**. The level of another non-cellulosic polysaccharide (e.g. starch) may be lower in the T-DNA tagged B14 population than Ws.

The presence of the T-DNA tag in the B14 population appears to have had no effect on the susceptibility of galacturonic acid, rhamnose, galactose, arabinose, mannose or glucuronic acid to the two screens. The results imply that there was less Driselase-susceptible fucose in the T-DNA tagged B14 population than Ws. This could be due to less fucose in the side chains of xyloglucan or rhamnogalacturonan.

4.3.4.5 Screen products for the *A. thaliana* line B16

Residue mass and mass of total identified products

None of the B16 individuals had TFA residues with masses significantly different from those of Ws (Table 4.5). However, 50% of B16 individuals had significantly larger residues (by 35-78%) than Ws. In the Driselase screen the total identified products of all the individuals were not significantly different from Ws and the masses of the digestion residues were significantly different from Ws in only 17% of individuals. These results indicate that there were no consistent differences between the T-DNA tagged B5 population and Ws in the susceptibility of the AIRs to the two screens.

Glucose

The proportion of glucose in the TFA products was significantly different from Ws in only 13% of individuals; while for the Driselase screen 50% of individuals had a significantly different proportion of glucose, however 33% were decreases and 17% increases (Tables 4.5 and 4.6). There was significantly more isoprimeverose than in Ws in 50% of B16 individuals. These results indicate that there was no difference between the T-DNA tagged B16 population and Ws in the level of cellulose- or non-cellulose-derived glucose.

Xylose

The proportions of xylobiose and isoprimeverose in the Driselase digest were significantly higher than in Ws in 50% of individuals, (Table 4.6). The proportion of xylose in the TFA products was only significantly different from Ws in 13% of individuals (Table 4.5). These results indicate that there were no consistent differences between the T-DNA tagged B16 population and Ws in the susceptibility of xylose to the two screens.

Galacturonic acid and rhamnose

The proportion of galacturonic acid in the Driselase products of B16 was significantly lower than in Ws in 33% of individuals; while in the TFA products the proportions were significantly higher than Ws for 38% of individuals. For rhamnose the proportions in the Driselase products were significantly lower than Ws for 17% of individuals and higher in 50% of individuals; and the proportions in the TFA products were significantly lower than in Ws, in 38% of individuals (Tables 4.5 and 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B16 population and Ws in the susceptibility of galacturonic acid or rhamnose to the two screens.

Galactose

The proportion of galactose in the TFA products was significantly higher than Ws in only 13% of individuals (Table 4.5). In the Driselase products the proportion of galactose was significantly lower (by 22-36%) than Ws in 83% of individuals (Table 4.6). This indicates that there was less Driselase-susceptible galactose in the T-DNA tagged B16 population than in Ws.

Arabinose

The proportion of arabinose in the TFA products was significantly higher than Ws, in only 25% of individuals. In the Driselase products 17% of individuals had significantly higher arabinose than Ws and 33% had significantly less. These results indicate that there were no consistent differences between the T-DNA tagged B16 population and Ws in the susceptibility of arabinose to the two screens (Tables 4.5 and 4.6).

Mannose

The proportion of mannose in the TFA products was significantly lower than in Ws, in 25% of individuals, while in the Driselase products the proportion was not significantly different from Ws for any of the individuals (Tables 4.5 and 4.6). This indicates that there were no consistent differences between the T-DNA tagged B16 population and Ws in the susceptibility of mannose to the two screens.

Fucose

The proportion of fucose in the TFA products was significantly different from Ws in only 13% of individuals, while in the Driselase products the proportion was significantly lower than in Ws, in 50% of individuals (Tables 4.5 and 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B16 population and Ws in the susceptibility of fucose to the two screens.

Glucuronic acid

The proportion of glucuronic acid in the Driselase products was significantly higher than in Ws, in 50% of individuals, while the proportion in the TFA products was significantly lower than Ws in 38% of individuals (Tables 4.5 and 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B5 population and Ws in the susceptibility of glucuronic acid to the two screens.

Summary

The results indicate that the presence of the T-DNA tag in the B16 population had no effect on the proportion of the AIRs that were susceptible to the two screens

The presence of the T-DNA tag in the B16 population had no effect on the amounts of cellulose-or non-cellulose-derived glucose in the AIRs, or the level of xylose susceptible to the two screens.

The presence of the T-DNA tag in the B16 population appears to have had no effect on the susceptibility of galacturonic acid, rhamnose, arabinose, mannose, fucose or glucuronic acid to the two screens. The results imply that there was less Driselase-susceptible galactose in the T-DNA tagged B16 population than Ws. This could be due to a change in the structure or composition of xyloglucan, the arabinogalactans or the oligosaccharide side-chains of glycoproteins.

4.3.4.6 Screen products for the *A. thaliana* line B17

Residue mass and mass of total identified products

For B17 the total masses of the identified products of the two screens were not significantly different from Ws for any of the individuals (Tables 4.5 and 4.6). There were no significant differences between Ws and B17 in the masses of the Driselase residues or the TFA hydrolysis residues. These

results indicate that there were no differences between the T-DNA tagged B17 population and Ws in the susceptibility of the AIRs to the two screens.

Glucose

In the Driselase screen the proportion of glucose was significantly higher (by 15-35%) than Ws in 60% of individuals (Table 4.6). The proportion of glucose in the TFA products was significantly different from Ws in only 14% of individuals (Table 4.5). This could indicate that the higher Driselase-susceptible glucose was due to cellulose-derived glucose. This could be caused by a recessive mutation as the percentage of the B17 population showing the significant difference was approximately 58%. The proportion of isoprimeverose was significantly different from Ws in 40% of B17 individuals, although half were higher than Ws and half lower. This indicates that there were no consistent differences between the T-DNA tagged B17 population and Ws in the susceptibility of xyloglucan to the Driselase screens.

Xylose

In the TFA screen only 14% of individuals had a significantly different proportion of xylose than Ws, while in the Driselase screen none of the individuals showed a significant difference from Ws (Tables 4.5 and 4.6). The proportions of isoprimeverose and xylobiose were significantly different from Ws in only 40% of individuals, and in both cases half were higher than Ws and half lower. These results indicate that there were no consistent differences between the T-DNA tagged B17 population and Ws in the susceptibility of xylose to the two screens.

Galacturonic acid and rhamnose

The proportion of galacturonic acid in the Driselase products was significantly lower (by 23-80%) than Ws for 80% of individuals, while in the TFA products the proportion was only significantly different in 29% of

individuals. The proportion of rhamnose in the Driselase products was significantly different from Ws in only 20% of individuals but in the TFA screen 57% of individuals had significantly less rhamnose (by 25-33%) than Ws (Tables 4.5 and 4.6).

These results indicate there was less Driselase-susceptible galacturonic acid and less TFA-susceptible rhamnose in the T-DNA tagged B17 population than in Ws.

Galactose

There were no significant differences between Ws and B17 in the proportion of galactose in the Driselase products. In the TFA screen only 29% of individuals had significantly more galactose than Ws (Tables 4.5 and 4.6). These results indicate that there were no consistent differences between the T-DNA tagged B17 population and Ws in the susceptibility of galactose to the two screens.

Arabinose

The proportions of arabinose in the TFA and Driselase products were significantly higher than in Ws, in 29% and 40% of individuals, respectively (Tables 4.5 and 4.6). This indicated that there were no consistent differences between the T-DNA tagged B17 population and Ws in the proportion of arabinose susceptible to the two screens.

Mannose

There were no significant differences between B17 and Ws in the proportion of mannose present in the products of either of the two screens (Tables 4.5 and 4.6). These results indicate that there were no differences between the T-DNA tagged B17 population and Ws in the susceptibility of mannose to the two screens.

Fucose

The proportion of fucose in the TFA products was significantly different from Ws in only 14% of individuals, while in the Driselase products the proportion was significantly lower (by 33-44%) than Ws in 60% of individuals (Tables 4.5 and 4.6). This would appear to indicate that there was less Driselase-susceptible fucose in the T-DNA tagged B17 population than in Ws.

Glucuronic acid

The proportion of glucuronic acid in the Driselase and TFA screens were significantly lower than in Ws, in 40% and 57% of individuals, respectively (Tables 4.5 and 4.6). These results indicate that there was possibly less TFA-susceptible glucuronic acid in the T-DNA tagged B17 population than in Ws.

Summary

The results indicate that the presence of the T-DNA tag in the B17 population had no effect on the proportion of the AIRs that were susceptible to the two screens

The T-DNA tagged B17 population had more cellulose-derived glucose than Ws, possible caused by a recessive mutation. There were no consistent differences between Ws and the B17 T-DNA tagged population in the amount of xyloglucan-derived glucose or the amount of xylose susceptible to the two screens.

The presence of the T-DNA tag in the B17 population appears to have had no effect on the susceptibility of galactose, arabinose or mannose to the two screens. The results imply that there was less Driselase-susceptible galacturonic acid and fucose, and less TFA-susceptible rhamnose and glucuronic acid in the T-DNA tagged B17 population than Ws. This could be due to a change in the structure or composition of the pectic polysaccharides (galacturonic acid, rhamnose, fucose, glucuronic acid),

xyloglucan (fucose) or xylan (glucuronic acid). The differences in rhamnose, fucose and glucuronic acid may be due to a recessive mutation as the percentages of the population which showed the significant difference were approximately 58% (57% for rhamnose and glucuronic acid and 60% for fucose).

4.3.4.7 Screen products for the *A. thaliana* line B24

Residue mass and mass of total identified products

The total mass of identified products for the B24 individuals were not significantly different from Ws for either TFA hydrolysis or Driselase digestion (Tables 4.5 and 4.6). The Driselase and TFA residue masses were significantly different from Ws in 71% and 67% of B24 individuals, respectively. In both cases there were both increases (57% for Driselase and 17% for TFA) and decreases (14% for Driselase and 50% for TFA) compared to Ws. These results indicate that there were no consistent differences between the T-DNA tagged B24 population and Ws in the susceptibility of the AIRs to the TFA screen. However there was possibly a difference between the T-DNA tagged B24 population and Ws in the susceptibility of the AIRs to Driselase digestion.

Glucose

The proportion of glucose in the Driselase digest was significantly higher (by 12-38%) than in Ws in 86% of B24 individuals. In the TFA hydrolysate the proportion of glucose was not significantly different from Ws for any of the individuals (Tables 4.5 and 4.6). This indicated that the significantly higher proportion of Driselase-susceptible glucose was due to a higher level of cellulose present in the T-DNA tagged B24 population than in Ws.

The proportion of isoprimeverose was significantly different from Ws in only 29% of individuals indicating that there was no difference between

Ws and the T-DNA tagged B24 population in the level of xyloglucan present.

Xylose

The proportion of xylobiose in the Driselase products was significantly lower (by 25-65%) than Ws in 86% of B24 individuals. This indicated that there was a lower proportion of Driselase-susceptible xylan in the T-DNA tagged B24 population. A lower proportion of xylan in the AIRs of B24 would be expected to give a lower proportion of xylose in the TFA hydrolysate, and the xylose proportion was significantly lower (by 38-67%) than in Ws in 50% of B24 individuals (Table 4.5).

As stated above only 28% of B24 individuals had a proportion of isoprimeverose significantly different from Ws (Table 4.6), indicating that the proportion of Driselase-susceptible xyloglucan in the T-DNA tagged B24 population was approximately the same as Ws.

The free xylose in the Driselase digest was derived from xylan and the side chains of rhamnogalacturonan. Only 14% of individuals had a significantly different proportion of xylose in the Driselase products.

Galacturonic acid and rhamnose

The proportion of galacturonic acid in the Driselase screen was significantly different from Ws in only 14% of individuals. In the TFA screen 50% of individuals had a significantly lower proportion of galacturonic acid (by 25-40%) than Ws. The proportion of rhamnose in the Driselase screen was significantly higher (by 35-135%) than Ws in 71% of individuals, while in the TFA screen only 17% of individuals had a significant difference from Ws. This could indicate a higher level of Driselase-susceptible rhamnose in the T-DNA tagged B24 population than in Ws.

Galactose

The proportion of galactose in the TFA products of B24 was significantly higher (by 72-155%) than in Ws in 67% of individuals (Table

4.5). In the Driselase products the proportion was significantly lower in 43% of individuals (Table 4.6). This indicates a difference between the T-DNA tagged B24 population and Ws in the susceptibility of galactose to the two screens. These results could be due to an increased susceptibility of arabinogalactans to TFA.

Arabinose

The proportion of arabinose in the TFA products was significantly higher (by 42-126%) than in Ws in 67% of individuals (Table 4.5). In the Driselase products the proportion was significantly lower in 43% of individuals (Table 4.6). This could indicate a difference between the T-DNA tagged B24 population and Ws in the susceptibility of arabinose to the two screens. These results may be due to an increased susceptibility of arabinogalactans to TFA.

Mannose

The proportions of mannose in the TFA and Driselase screens were not significantly different from Ws for any of the B24 individuals (Tables 4.5 and 4.6). This implies that there was no difference between the T-DNA tagged B24 population and Ws in the susceptibility of mannose to the two screens.

Fucose

The proportion of fucose in the TFA products was significantly higher (by 50-63%) than in Ws in 50% of individuals (Table 4.5). In the Driselase products the proportion of fucose was significantly less than Ws in only 14% of individuals (Table 4.6). This probably indicates that there was no difference between the T-DNA tagged B24 population and Ws in the susceptibility of fucose to the two screens.

Glucuronic acid

The proportion of glucuronic acid in the TFA products was significantly lower than Ws in only 17% of individuals, while in the Driselase products the proportion was significantly lower (by 100%) in 57% of individuals (Tables 4.5 and 4.6). These results indicate that there was a lower proportion of Driselase-susceptible glucuronic acid in the T-DNA tagged B24 population than Ws.

Summary

The results indicate that the presence of the T-DNA tag in the B24 population could possibly have reduced the Driselase-susceptibility of the AIRs. This could indicate the increased presence of a Driselase-resistant polymer in the AIR.

The T-DNA tagged B24 population had more cellulose-derived glucose and less Driselase-susceptible xylan than Ws. The difference between Ws and the T-DNA tagged B24 population in the Driselase-susceptibility of xylan could be due to a higher level of substituted xylose residues (with [methyl]glucuronic acid residues, arabinose residues and acetyl groups) in B24 than Ws. The presence of the T-DNA tag in the B24 population appears to have had no effect on the level of Driselase-susceptible xyloglucan.

The T-DNA tagged B24 population has more Driselase-susceptible rhamnose, more TFA-susceptible galactose and arabinose and less Driselase-susceptible glucuronic acid than Ws. These results could be due to changes in the structure and/ or the substitution pattern of the pectic polysaccharides (rhamnose), arabinogalactans (galactose and arabinose), xyloglucan (galactose), xylan (arabinose and glucuronic acid) or the oligosaccharide side-chains of glycoproteins (galactose and arabinose). The differences in galactose, arabinose and glucuronic acid may be due to a recessive mutation(s) as the percentages of the population which showed the

significant difference were less than 75% (67% for galactose and arabinose and 57% for fucose).

The presence of the T-DNA tag in the B24 population appears to have had no effect on the susceptibility of galacturonic acid, mannose or fucose to the two screens.

4.3.4.8 Screen products for the *A. thaliana* line B27

Residue mass and mass of total identified products

In the TFA screen 40% of B27 individuals had a significantly lower mass of total products than Ws, 40% of individuals also had a significantly lower TFA residue mass (Table 4.5). The results of the Driselase screen indicated that while there were no significant differences between Ws and the B27 individuals in the total mass of identified carbohydrate products, 60% of B27 individuals did have a significantly heavier residue than Ws (by 21-34%; Table 4.6). These results indicate that a smaller proportion of the AIR of B24 was susceptible to Driselase digestion but approximately the same masses of products were identified. This implies that there was a lower level of unidentified products from the T-DNA tagged B27 population compared to Ws.

Glucose

The proportion of glucose in the Driselase digests of the B27 individuals was significantly higher (by 20-37%) than Ws in 80% of individuals (Table 4.6). There were no significant differences between the B27 individuals and Ws in the proportion of glucose in the TFA hydrolysate (Table 4.5), which implies that the differences in the proportion of glucose in the Driselase digests was due to an increased breakdown of cellulose by Driselase. The proportion of isoprimeverose was significantly less than Ws for only 40% of B27 individuals, indicating no difference between Ws and

the T-DNA tagged B27 population in the level of Driselase-susceptible xyloglucan.

Xylose

The proportion of xylobiose in the Driselase screen was significantly lower (by 58-68%) than in Ws in 80% of the individuals. The proportion of xylose in the TFA hydrolysate was significantly lower in only 40% of individuals (Table 4.5). These results indicate that the total amount of xylose present in the T-DNA tagged B27 population was not different from Ws but the proportion present in Driselase-susceptible xylan was lower. This could be due to a higher level of substituted (with [methyl]glucuronic acid residues, arabinose residues, and acetyl groups) xylose residues.

In the Driselase products, 60% of B27 individuals had significantly less xylose (by 37-50%) than Ws. The free xylose in the Driselase products was derived from xylan and the side chains of rhamnogalacturonan.

As stated above there was no difference between the T-DNA tagged B27 population and Ws in the level of Driselase-susceptible xyloglucan.

Galacturonic acid and rhamnose

The proportions of galacturonic acid in the products of both screens were significantly different than Ws in 40% of individuals for both TFA hydrolysis and Driselase digestion (Tables 4.5 and 4.6). Although in the Driselase screen 20% had significantly more galacturonic acid than Ws and 20% significantly less. The proportions of rhamnose in the TFA products were not significantly different from Ws for any of the B27 individuals, while in the Driselase products the proportions were significantly lower than Ws in 40% of individuals. These results indicate no difference between Ws and the T-DNA tagged B27 population in the susceptibility of galacturonic acid and rhamnose to the two screens.

Galactose

The proportion of galactose in the TFA products was significantly higher (by 34-56%) than Ws in 80% of B27 individuals (Table 4.5) and in the Driselase products the proportion was significantly lower (by 32-36%) than Ws in 80% of individuals (Table 4.6). This indicates that there was more TFA-susceptible and less Driselase-susceptible galactose in the T-DNA tagged B27 population than in Ws.

Arabinose

The proportion of arabinose in the TFA products was significantly higher (by 69-161%) than Ws in 80% of B27 individuals (Table 4.5) and in the Driselase products the proportion was significantly lower (by 29-57%) than Ws in 80% of individuals (Table 4.6). This indicates that there was more TFA-susceptible and less Driselase-susceptible arabinose in the T-DNA tagged B27 population than in Ws.

Mannose

The proportion of mannose in the TFA products was not significantly different from Ws in any of the B27 individuals (Table 4.5). In the Driselase products the proportion was significantly lower (by 60-72%) than Ws in 60% of individuals (Table 4.6). This could indicate a lower proportion of Driselase-susceptible mannose in the T-DNA tagged B27 population than in Ws.

Fucose

The proportion of fucose in the TFA products was significantly higher than Ws in only 40% of individuals (Table 4.5). In the Driselase products the proportion was significantly lower (by 22-44%) in 80% of individuals (Table 4.6). This could indicate less Driselase-susceptible fucose in the T-DNA tagged B27 population than in Ws.

Glucuronic acid

The proportion of glucuronic acid was significantly lower than Ws (by 50-57%) in 60% of B27 individuals for the TFA hydrolysate and (by 67-100%) in 80% of individuals for the Driselase digest (Tables 4.5 and 4.6). As the decrease was observed in the products of both screens, it is possible that this indicates a decreased proportion of glucuronic acid in the pectins.

Summary

The results indicate that the presence of the T-DNA tag in the B27 population had no effect on the proportion of the AIRs that were susceptible to the TFA screen. However, there was a lower level of unidentified Driselase products from the T-DNA tagged B27 population than Ws. This could be due to a recessive mutation as the percentage of the population showing the significant difference was 60%.

The T-DNA tagged B27 population had more cellulose-derived glucose and less Driselase-susceptible xylan than Ws. The difference between Ws and the T-DNA tagged B27 population in the Driselase-susceptibility of xylan could be due to a higher level of substituted xylose residues (with [methyl]glucuronic acid residues, arabinose residues and acetyl groups) in B27 than Ws. The presence of the T-DNA tag in the B27 population appears to have had no effect on the level of Driselase-susceptible xyloglucan.

The T-DNA tagged B27 population had less free xylose in the Driselase products than Ws. This could be due to a recessive mutation as the percentage of the population showing the significant difference was 60%, and may reflect a change in the composition and/ or structure of xylan or rhamnogalacturonan.

The T-DNA tagged B27 population has more TFA-susceptible and less Driselase-susceptible galactose and arabinose, less Driselase-susceptible mannose and fucose, and less Driselase- and TFA-susceptible glucuronic

acid than Ws. These results could be due to changes in the structure and/ or the substitution pattern of the pectic polysaccharides (glucuronic acid), arabinogalactans (galactose and arabinose), xyloglucan (galactose), xylan (arabinose), glucomannans (mannose) or the oligosaccharide side-chains of glycoproteins (mannose, galactose and arabinose). The differences in mannose and TFA-susceptible glucuronic acid may be due to a recessive mutation(s) as the percentages of the population which showed the significant difference were less than 75% (60% for both mannose and glucuronic acid).

The presence of the T-DNA tag in the B27 population appears to have had no effect on the susceptibility of galacturonic acid, mannose or fucose to the two screens.

4.3.4.9 Screen products for the *A. thaliana* line B34

Residue mass and mass of total identified products

The mass of residues and total identified products of TFA hydrolysis were significantly different from Ws for 17% and 33% of the B34 individuals, respectively (Table 4.5). In the Driselase screen, none of the B34 individuals had a significant difference from Ws in the total mass of identified products but 38% of the individuals did have a significantly lower residue mass (Table 4.6). These results indicated that the proportions of the AIRs of the T-DNA tagged B34 population solubilised by the two screens were not different from Ws.

Glucose

The proportion of glucose in the Driselase products of B34 was significantly higher (by 11-36%) than Ws for 75% of B34 individuals (Table 4.6). The proportion of glucose in the TFA products was significantly different from Ws for only 17% of the B34 individuals (Table 4.5). This indicated that the higher proportion of Driselase-susceptible glucose was

due to a higher level of Driselase-susceptible cellulose in the T-DNA tagged B34 population compared to Ws. The proportion of isoprimeverose in the Driselase digests was significantly lower (by 18-32%) than Ws in 88% of B34 individuals (Table 4.6). This implies that there was less Driselase-susceptible xyloglucan in the T-DNA tagged B34 population than in Ws.

Xylose

The proportions of xylose in the TFA and Driselase products were significantly less than Ws for only 33% and 25% of B34 individuals, respectively (Tables 4.5 and 4.6). The proportion of xylobiose in the Driselase products was significantly lower than Ws in only 38% of B34 individuals, indicating no overall difference between the T-DNA tagged B34 population and Ws in the Driselase-susceptibility of xylan. As stated above, the results indicate there was less Driselase-susceptible xyloglucan in the T-DNA tagged B34 population than in Ws.

Galacturonic acid and rhamnose

The proportions of rhamnose in the TFA and Driselase products were significantly lower than Ws in only 33% and 13% of B34 individuals, respectively (Tables 4.5 and 4.6). The proportion of galacturonic acid in the TFA products was significantly higher than Ws in 33% of B34 individuals, while the proportion in the Driselase products was significantly lower in 38% of individuals. These results indicate that there was no difference between Ws and the T-DNA tagged B34 population in the susceptibility of galacturonic acid and rhamnose to the two screens.

Galactose

The proportion of galactose in the TFA products was significantly higher (by 28-100%) than Ws in 50% of B34 individuals (Table 4.5). In the Driselase products the proportion was significantly lower in only 38% of individuals (Table 4.6). This indicates that there was no difference between

the T-DNA tagged B34 population and Ws in the susceptibility of galactose to the two screens.

Arabinose

The proportion of arabinose in the TFA products was significantly higher (by 35-117%) than Ws in 50% of B34 individuals (Table 4.5). In the Driselase products the proportion was significantly lower (by 29-35%) in 50% of individuals (Table 4.6). This indicates that there was no difference between the T-DNA tagged B34 population and Ws in the susceptibility of arabinose to the two screens.

Mannose

There were no significant differences between Ws and the T-DNA tagged B34 population in the proportions of mannose present in the products of the two screens (Tables 4.5 and 4.6).

Fucose

The proportion of fucose in the TFA products was significantly higher than Ws in only 33% of B34 individuals (Table 4.5). In the Driselase products the proportion was significantly lower (by 22-56%) in 63% of individuals (Table 4.6). This could indicate a difference between the T-DNA tagged B34 population and Ws in the Driselase-susceptibility of fucose.

Glucuronic acid

The proportions of glucuronic acid in the Driselase and TFA products were significantly lower than Ws (by 67-100%) in 63% and (by 50-86%) in 100% of B34 individuals, respectively (Tables 4.5 and 4.6). As lower proportions of glucuronic acid were observed in the products of both screens, it is possible that this indicates a lower proportion of glucuronic acid in the pectins of the T-DNA tagged B34 population than Ws.

Summary

The results indicate that the presence of the T-DNA tag in the B34 population had no effect on the proportion of the AIRs that were susceptible to the two screens

The T-DNA tagged B34 population had more cellulose-derived glucose and less Driselase-susceptible xyloglucan than Ws. The presence of the T-DNA tag in the B34 population appears to have had no effect on the level of Driselase-susceptible xylan.

The T-DNA tagged B34 population had less TFA- and Driselase-susceptible glucuronic acid and less Driselase-susceptible fucose than Ws. These results could be due to changes in the structure and/ or the substitution pattern of the pectic polysaccharides (fucose or glucuronic acid) or xylan (glucuronic acid). The differences in Driselase-susceptible fucose and glucuronic acid may be due to a recessive mutation as the percentages of the population that showed the significant differences were less than 75%.

The presence of the T-DNA tag in the B34 population appears to have had no effect on the susceptibility of galacturonic acid, rhamnose, galactose, arabinose and mannose to the two screens.

4.3.4.10 Screen products for the *A. thaliana* line B52

Residue mass and mass of total identified products

For B52 the mass of the Driselase digestion residue was significantly lower (by 23-50%) than Ws in 57% of individuals and higher in 14% (Table 4.6). The mass of the TFA hydrolysis residue and the total masses of identified Driselase carbohydrate products were not significantly different from those of Ws for any of the B52 individuals (Tables 4.5 and 4.6). The total identified products of the TFA screen were significantly different from Ws in only 29% of B52 individuals. These results imply that the increased

solubilisation of the AIRs of the T-DNA tagged B52 population by Driselase was due to unidentified products.

Glucose

The proportion of the TFA products that was glucose was not significantly different from Ws for any of the B52 individuals. In the Driselase products the proportions of glucose and isoprimeverose were significantly different from Ws in 57% of individuals, in both cases (Table 4.6). However, 43% of individuals had lower glucose than Ws and 14% higher, while 14% of individuals had lower isoprimeverose than Ws and 43% higher. These results appear to indicate that there were no consistent differences between the T-DNA tagged B52 population and Ws in the susceptibility of glucose to the two screens.

Xylose

The proportion of xylose in the TFA hydrolysate was significantly lower (by 27-37%) than Ws in 71% of B52 individuals, while the proportion of free xylose in the Driselase digest was significantly higher than Ws in only 29% of individuals (Tables 4.5 and 4.6). The proportion of xylobiose was significantly higher (by 39-51%) than Ws in 57% of B52 individuals. This could indicate a higher level of Driselase-susceptible xylan in the T-DNA tagged B52 population than Ws. As stated above there was no difference between the T-DNA tagged B52 population and Ws in the level of Driselase-susceptible xyloglucan.

Galacturonic acid and rhamnose

For the TFA and Driselase screens the proportions of rhamnose were significantly different from Ws in only 14% and 29% of B52 individuals, respectively (Tables 4.5 and 4.6). The proportion of galacturonic acid in the TFA products was significantly higher than Ws in 43% of individuals, while the proportion in the Driselase products was significantly lower than Ws in

only 14% of individuals. These results indicate that there were no consistent differences between the T-DNA tagged B52 population and Ws in the susceptibility of galacturonic acid and rhamnose to the two screens.

Galactose

The proportion of galactose in the TFA products was significantly higher (by 27-67%) than Ws in 86% of B52 individuals (Table 4.5) and for the Driselase screen significantly lower than Ws in only 43% of individuals (Table 4.6). This indicated that there was more TFA-susceptible galactose in the T-DNA tagged B52 population than Ws.

Arabinose

The proportion of arabinose in the TFA products was significantly different from Ws for only 14% of the B52 individuals (Table 4.5). In the Driselase products the proportion was significantly different from Ws in only 14% of individuals (Table 4.6). This implies that there were no consistent differences between Ws and the T-DNA tagged B52 population in the susceptibility of arabinose to the two screens.

Mannose

There were no significant differences between Ws and the T-DNA tagged B52 population in the proportions of mannose present in the products of the two screens (Tables 4.5 and 4.6).

Fucose

The proportion of fucose in the TFA products was significantly higher (by 25-63%) than Ws in 71% of B52 individuals (Table 4.5), while in the Driselase products the proportion was significantly lower (by 22-44%) in 57% of individuals and higher in 14% (Table 4.6). These results could indicate a difference between the T-DNA tagged B52 population and Ws in the susceptibility of fucose to the two screens.

Glucuronic acid

The proportion of glucuronic acid in the Driselase products was significantly higher than Ws in 43% of B52 individuals and significantly lower in 29% of individuals. In the TFA products the proportion was significantly lower (by 43-57%) than Ws in 86% of individuals (Table 4.5). As the difference was only observed in the TFA products it is possible that there was less glucuronic acid present in xylan in the T-DNA tagged B52 population than Ws.

Summary

The results indicate that the presence of the T-DNA tag in the B52 population had no effect on the proportion of the AIRs that were susceptible to the TFA screen. However, there was a higher level of unidentified Driselase products from the T-DNA tagged B52 population than Ws. This could be due to a recessive mutation as the percentage of the population showing the significant difference was 57%.

The presence of the T-DNA tag in the B52 population appears to have had no effect on the level of cellulose- or non-cellulose-derived glucose. **The T-DNA tagged B52 population had less xylan than Ws, and a higher proportion of it was Driselase-susceptible. The difference between Ws and the T-DNA tagged B52 population in the Driselase-susceptibility of xylan could be due to a lower level of substituted xylose residues (with [methyl]glucuronic acid residues, arabinose residues and acetyl groups) in B52 than Ws. This could be due to a recessive mutation as the percentage of the population that showed the significant difference was less than 75%.**

The T-DNA tagged B52 population had more TFA-susceptible galactose, less TFA-susceptible glucuronic acid and more TFA- and less Driselase-susceptible fucose than Ws. These results could be due to changes in the structure and/ or the substitution pattern of the arabinogalactans

(galactose), xyloglucan (galactose), xylan (glucuronic acid), xyloglucan (galactose and fucose), rhamnogalacturonan (fucose) or the oligosaccharide side-chains of glycoproteins (galactose).

The presence of the T-DNA tag in the B52 population appears to have had no effect on the susceptibility of galacturonic acid, rhamnose, arabinose and mannose to the two screens.

4.3.4.11 Screen products for the *A. thaliana* line B58

Residue mass and mass of total identified products

There were no significant differences between Ws and any of the B58 individuals in the total identified Driselase products (Tables 4.5 and 4.6). The Driselase residue was significantly smaller (by 21-39%) than Ws for 60% of the B58 individuals and larger in 20% (Table 4.6). For the TFA screen the total mass of identified products and the residue mass were significantly lower than in Ws in 50% and 33%, respectively, of B58 individuals. These results imply that the increased solubilisation of the AIRs of the T-DNA tagged B58 population by Driselase was due to unidentified products.

Glucose

The proportion of glucose in the TFA hydrolysate was significantly lower than Ws in only 17% of B58 individuals (Table 4.5). In the Driselase products 60% of B58 individuals had a significantly higher proportion of glucose (by 10-18%) and only 40% had a significantly different proportion of isoprimeverose (20% higher and 20% lower, Table 4.6). These results indicate there was more cellulose-derived glucose and approximately the same level of xyloglucan-derived glucose in the Driselase products of the T-DNA tagged B58 population than Ws.

Xylose

The proportion of xylose in the TFA products was significantly lower (by 45-68%) than Ws in 50% of B58 individuals (Table 4.5). There were no significant differences between Ws and any of the B58 individuals in the proportion of xylose in the Driselase products (Table 4.6). The proportion of xylobiose was significantly higher (by 39-46%) than Ws in 80% of B58 individuals, indicating a higher level of Driselase-susceptible xylan than in Ws. These results indicated that there was a large difference between Ws and the T-DNA tagged B58 population in the total yield of xylose present, but the degree of substitution of xylose residues (with [methyl]glucuronic acid residues, arabinose residues and acetyl groups) was higher in the T-DNA tagged B58 population than Ws.

As stated above, there were no differences between Ws and the T-DNA tagged B58 population in the level of Driselase-susceptible xyloglucan.

Galacturonic acid and rhamnose

The proportions of rhamnose in the TFA and Driselase products were not significantly different from Ws for any of the B58 individuals (Tables 4.5 and 4.6). Galacturonic acid was significantly higher (by 16-40%) than Ws in the TFA products of 50% of individuals and significantly lower in the Driselase products of 40% of individuals. These results indicate that there were no consistent differences between the T-DNA tagged B58 population and Ws in the susceptibility of galacturonic acid and rhamnose to the two screens.

Galactose

The proportions of galactose in the TFA products were significantly higher (by 27-138%) than Ws in 66% of individuals (Table 4.5). In the Driselase products the proportions were significantly lower in only 20% of individuals (Table 4.6). This implies that there was more TFA-susceptible galactose in the T-DNA tagged B58 population than Ws.

Arabinose

The proportions of arabinose in the TFA products were significantly higher (by 26-164%) than Ws in 83% of individuals (Table 4.5). In the Driselase products the proportions were not significantly different from Ws for any of the B58 individuals (Table 4.6). This indicates that there is more TFA-susceptible arabinose in the AIRs of B58 than Ws.

Mannose

The proportions of mannose in the TFA products were significantly lower than Ws in only 17% of individuals (Table 4.5). In the Driselase products the proportions were not significantly different from Ws in any of the B58 individuals (Table 4.6). This indicates that there were no consistent differences between the T-DNA tagged B58 population and Ws in the susceptibility of mannose to the two screens.

Fucose

The proportions of fucose in the TFA products were significantly higher (by 50-75%) than Ws in 50% of B58 individuals (Table 4.5). In the Driselase products the proportions were significantly lower in 40% of individuals (Table 4.6). This indicates that there were no consistent differences between the T-DNA tagged B58 population and Ws in the susceptibility of fucose to the two screens.

Glucuronic acid

The proportions of glucuronic acid in the Driselase products were significantly lower than Ws in 60% of B58 individuals. In the TFA products the proportions were significantly lower (by 36-86%) than Ws in 83% of individuals (Table 4.6). These results imply that there was less glucuronic acid present in the AIRs of the T-DNA tagged B58 population that was susceptible to the two screens.

Summary

The results indicate that the presence of the T-DNA tag in the B58 population had no effect on the proportion of the AIRs that were susceptible to the TFA screen. However, there was a higher level of unidentified Driselase products from the T-DNA tagged B58 population than Ws. This could be due to a recessive mutation as the percentage of the population showing the significant difference was 60%.

The T-DNA tagged B58 population had more cellulose-derived glucose than Ws. This could be due to a recessive mutation as the percentage of the population that showed the significant difference was less than 75%. There was more Driselase-susceptible xylan in the T-DNA tagged B58 population than Ws. The difference between Ws and the T-DNA tagged B58 population in the Driselase-susceptibility of xylan could be due to a lower level of substituted xylose residues (with [methyl]glucuronic acid residues, arabinose residues and acetyl groups) in B58 than Ws. The presence of the T-DNA tag in the B58 population appeared to have no effect on the susceptibility of xyloglucan to the two screens.

The T-DNA tagged B58 population had more TFA-susceptible galactose and arabinose and less TFA- and Driselase-susceptible glucuronic acid than in Ws. These results could be due to changes in the structure and/ or the substitution pattern of the arabinogalactans (galactose and arabinose), xyloglucan (galactose), xylan (arabinose and glucuronic acid), pectins (arabinose, galactose and glucuronic acid) or the oligosaccharide side-chains of glycoproteins (galactose and arabinose). The differences in TFA-susceptible galactose and Driselase-susceptible glucuronic acid could be due to a recessive mutation as the percentages of the population that showed the significant differences were less than 75%.

The presence of the T-DNA tag in the B58 population appears to have had no effect on the susceptibility of galacturonic acid, rhamnose, mannose or fucose to the two screens.

Table 4.11 Summary of the statistically significant differences from Ws, which may be due to the presence of a T-DNA tag in the *A. thaliana* line. Only differences from Ws in the cell wall polysaccharides which have an effect on paper quality are shown (Section 1.4).

Feldmann lines		Summary of statistically significant differences from Ws			
Screen No.	NASC No.	Cellulose	Xyloglucan	Xylan	Glucomannan
B5	N2848	-	more Driselase-susceptible xyloglucan	more Driselase-susceptible xylan	-
B14	N2858	-	more Driselase-susceptible xyloglucan	-	-
B16	N2860	-	-	-	-
B17	N2861	more cellulose-derived glc	-	-	-
B24	N2868	more cellulose-derived glc	-	less Driselase-susceptible xylan	-
B27	N2871	more cellulose-derived glc	-	less Driselase-susceptible xylan	less Driselase-susceptible mannose
B34	N2878	more cellulose-derived glc	less Driselase-susceptible xyloglucan	-	-
B52	N2896	-	-	less xylan and more of it Driselase-susceptible	-
B58	N2902	more cellulose-derived glc	-	more Driselase-susceptible xylan	-

5 General Results

5.1 Yield of alcohol-insoluble residue

Ethanol extraction of plant tissue (Section 2.2.3.3) gives a residue composed of cell wall polysaccharides and other polymers (e.g. RNA, starch and protoplasmic proteins). The ratio of AIR to fresh weight (FW) of the tissue (mg AIR/ g FW) gives an indication of the proportion of polymers in the flowering stem tissue.

All the plants were grown under the same growth conditions (Section 2.2.2.2) and the bolting stems were cut once the first siliques had formed so all the tissue used was at the same developmental stage.

The *A. thaliana* ecotypes Ws and Columbia had significantly different AIR: FW ratios (

Table 5.1). The yield of AIR for Columbia was 140% that of Ws. This implies that there was a greater proportion of polymers in the flowering stem of Columbia (a difference of 32.8 mg/ g FW) than there was in the flowering stem of Ws.

Of the nine Feldmann lines screened by HPLC, two had ratios significantly different from Ws (the Feldmann parental line). The AIR of B5 was 15% less than in Ws, while the AIR of B14 was 17% more than in Ws.

5.1.1 Discussion

There was no difference between Columbia and Ws (Section 4.3.4.2.), or B14 and Ws (Section 4.3.4.3), in the total mass of identified products or the mass of the residues of either screen, which could indicate that polymers resistant to the TFA and Driselase screens made up a larger proportion of the AIRs of Columbia and B14, than in Ws. Polymers present in the AIR that would be resistant to the two screens would be a proportion of the RNA and

protoplasmic proteins as well, and possibly a small percentage of the polysaccharides.

There could be a number of possible reasons for these differences. There could be differences in the thickness of the cell walls or there could be a higher proportion of any of the wall polysaccharides or there could be a higher percentage of xylem in the tissue.

None of the other lines screened by HPLC differed significantly from the Ws AIR: FW ratio.

Table 5.1 The relative mass of the AIR of the flowering stems of *A. thaliana* lines. The AIR was prepared as described in Section 2.2.3.3. ** = Probability of less than 0.05 that the line was from the same population as the Ws; * = probability of $0.05 < p < 0.10$ that the line was from the same population as the parental line, Ws (as calculated by the anova method; Section 2.2.6).

Line	No. of replicates (n)	Mass of AIR (mg/ g FW) mean	(std dev)	AIR as % of Ws (AIR: FW ratio)
Ws	11	81.1	(7.7)	-
Col	11	113.9 **	(15.4)	140.4
B5	10	68.7 **	(13.5)	84.7
B14	21	94.9 *	(24.0)	117.0
B16	19	80.6	(10.9)	99.4
B17	11	80.1	(13.1)	98.8
B24	18	83.2	(25.1)	102.6
B27	22	83.6	(34.9)	103.1
B34	22	87.6	(20.3)	108.0
B52	34	81.3	(26.8)	100.2
B58	13	76.2	(33.7)	94.0

5.2 Starch content of *A. thaliana AIR*

The AIR of *A. thaliana* ecotype Columbia (Section 2.2.3.3) was destarched by extraction in 90% DMSO (Section 2.1.5). The mass of the residue remaining after extraction of the starch was 90.2 ± 2.0% (n = 4) of that of the AIR. This suggests that the mass of starch extracted was approximately 10% of the mass of the AIR.

5.2.1 Discussion

Starch concentrations in plant tissue vary diurnally (Caspar, 1994) so the bolting stems of the *A. thaliana* lines were always harvested in the afternoons, although not at exactly the same time.

The DMSO extraction of Columbia AIR suggests that approximately 10% of the AIR mass was starch. As the AIR of Columbia was 11.4% of the fresh weight (Table 5.1), approximately 1.1% of the fresh weight of the bolting stem of Columbia was starch. However, DMSO extraction can also extract some acidic xylans (Fry, 1988).

In retrospect it would have been a good idea to destarch all plants that were screened prior to harvesting stems. However, the presence of starch would only affect the concentration of glucose observed. If the amount of starch was very much increased or decreased compared to the wild type then the proportions of the other components of the screen products would be altered; however, this could be taken into account when analysing the screen results.

5.3 Delignification of *A. thaliana* AIR

The AIR of *A. thaliana* ecotype Columbia (Section 2.3.3.3) and the DMSO extraction residue (Section 5.2) were delignified using the method of Whistler and BeMiller (1963).

The delignification treatment of the AIR extracted approximately 20% of the AIR mass. However, delignification of the destarched AIR (DMSO residue) only extracted the equivalent of 10% of the original AIR mass. It is possible that the treatment with sodium chlorite and acetic acid also extracted the starch from the AIR, hence the larger mass that was extracted from the untreated AIR.

Table 5.2 The mass of the delignified residues of untreated and destarched AIR. AIR was prepared as described in Section 2.3.3.3, and either extracted in DMSO (Section 2.1.5) or left untreated. The value for the mass of the residues are means (standard deviation in brackets); n= number of replicates; CWM = cell wall material

CWM	Residue mass (mg/ g CWM)	% of untreated AIR mass
untreated AIR	83	80.3%
n = 2	(55.6)	
destarched AIR	887	80.0%
n = 2	(6.8)	

5.3.1 Discussion

Ten percent of the mass of the destarched AIR of Columbia was extracted by the delignification method of Whistler and BeMiller (1963). This implies that approximately 10% of the AIR mass or 1% of the fresh weight of the bolting stem of Columbia is lignin. However, from the delignification of *Eucalyptus* spp. xylem using this method (Section 3.1.1) it would appear that not all of the lignin present was extracted. The lignin content of *A. thaliana* was investigated by Dharmawardhana (1992) and was found to be typical of that found in other angiosperms.

6 Genetic analysis of the *Arabidopsis* lines

All the lines identified by the screening methods were Feldmann lines from the NASC (Section 2.2.1.2 and Section 4). These lines were produced by transformation of *A. thaliana* (L.) Heynh ecotype Wassilewskija (Ws) with *Agrobacterium tumefaciens* strain C58C1rif containing the Ti plasmid 3850:1003 (Feldmann and Marks, 1987). The transformation event resulted in the insertion of the T-DNA region of the Ti plasmid into the nuclear genome of the plant (Feldmann, 1987; 1989).

The Ti plasmid 3850:1003 has the neomycin phosphotransferase gene (NPT II) contained between the two Ti plasmid borders. NPT II confers resistance to kanamycin, which interferes with protein synthesis. Wild type seedlings grown on media containing kanamycin will produce white leaves, while seedlings which have the NPT II gene will produce normal green leaves. Selection of resistant/ sensitive seedlings can be made on the basis of leaf colour.

The aim of this section was to establish whether resistance to kanamycin (i.e. the T-DNA tag) co-segregated with the observed differences from the wild type in the composition of the screen products (Section 4). The Feldmann lines were allowed to self and the seeds collected (F1, Section 2.3.1). F1 seeds were surface-sterilised and sown under aseptic conditions on agar plates containing kanamycin (Sections 2.2.2.2 and 2.3.2; Feldmann and Marks, 1987).

Two weeks after germination the resistant and sensitive seedlings were counted. There was a very low germination rate for some lines, and for some lines no seeds germinated. For the lines where a number of seeds germinated, the number of sensitive seedlings was far greater than the number of resistant seedlings. This was very suprising as kanamycin resistance is a dominant trait. The expected segregations of kanamycin resistance (Km^R) and sensitivity (Km^S) for tagged *Arabidopsis* lines with one T-DNA tag in their genome, are given in Table 6.1. The observed ratios of

Km^R to Km^S for the F1 of the Feldmann lines are given in Table 6.2. The observed ratios were completely different to the expected ratios. It is possible that the observed ratios were due to excess bleaching of the F1 seeds during seed sterilisation.

Two weeks after germination the sensitive seedlings were transferred to medium without kanamycin, so that they could be grown and tested for the predicted difference in the screen products. However, the sensitive plants did not grow normally once transplanted to agar and failed to produce bolting stems. The resistant seedlings were left on kanamycin-containing media; however, after some time, their leaves also became bleached.

Table 6.1 The theoretical percentages of Km^R and Km^S phenotypes in the F1 of the Feldmann lines

	% of F1 with Km^R/ Km^S phenotype if parent was:		
Phenotype	homozygous for wild type gene	heterozygous for NPT II gene	homozygous for NPT II gene
Km^R	0	75	100
Km^S	100	25	0

Table 6.2 Observed ratios of Km^R:Km^S in the F1 seeds of the T-DNA tagged *A. thaliana* lines.

			Number of seeds with phenotype:		
Line	No. of seeds sown	Proportion of seeds germinated	Km^R	Km^S	Ratio of Km^R: Km^S
B5	364	1.1%	-	4	-
B14	198	27.8%	5	50	1: 10
B16	721	52.2%	119	258	1: 2.2
B17	349	2.0%	-	7	-
B24	226	22.1%	-	50	-
B27	369	4.1%	-	15	-
B34	316	11.7%	-	37	-
B52	151	9.9%	3	12	1: 4
B58	200	33.5%	-	67	-

6.1 Discussion

It was not conclusively shown whether the T-DNA tag(s) of the identified lines co segregate with the observed differences in the screen products. An attempt was made to cross the lines back to the parental line in the hope of observing whether the T-DNA tag and the observed difference from the parental in the screen products co-segregated. This was not successful (two siliques began to develop but did not mature). Another attempt at back-crossing would have been made; however, this was not accomplished owing to lack of time.

7 Discussion

Eucalyptus wood is an economically important source of timber and pulp (Wilkins and Horne, 1991). The moderate requirements of the genus and the fast growth have contributed to making eucalypts one of the world's most important groups of plantation hardwoods (Wilkins and Horne, 1991).

Processing of pulp to produce paper and acetate products (such as cellophane and viscose rayons) involves several bleaching steps to remove all residual lignin. The by-products of these steps are chlorinated organic compounds which are known to have a toxic effect on the environment (Farrel and Skerker, 1992; Milagnes and Duran, 1992; Nissen *et al.*, 1992). Treatment of the pulp with xylanases has been shown to reduce the amount of chlorine required to bleach the pulp to the desired brightness indicating the importance of the lignin-xylan bond in pulp processing (Wong and Saddler, 1993).

The presence of some xylans and glucomannans has beneficial effects on paper properties such as increasing the tensile and bursting strength and giving improved folding endurance (Wise and Lauer, 1962; Gamerith and Strutzenberger, 1992). However, hemicelluloses are impurities in the production of acetate products as they cause defects in the final products and can damage machinery.

The ability to down-regulate the synthesis of specific hemicelluloses or to change their structure (so that they are easier to extract or bind less strongly to lignin) would be of benefit to the paper and pulp industries.

7.1 Analysis of *Eucalyptus* wood and pulp

The pulp produced from eucalypt wood and the xylem from two *Eucalyptus* species (*E. grandis* and *E. globulus*) were analysed to establish the polysaccharides present in the pulp used in paper production and also the levels of these polysaccharides in the xylem of *Eucalyptus* spp.

7.1.1 Xylem

There is some variation between the two eucalypt species in the level of substitution of the xylan backbone with (methyl)glucuronic acid and acetyl groups. The presence of acetyl groups on the xylose residues of the xylan backbone can affect the structure of the xylan molecule and hence the properties of the pulp produced from the walls. Decreased substitution of the xylan molecules allows them to hydrogen bond to cellulose microfibrils in the pulp (Varner and Lin, 1989) and makes them more difficult to extract (Joseleau *et al.*, 1992). Whether the observed variation between the two eucalypt species is enough to affect the properties of pulp made from these two species is not known.

Comparisons between *E. grandis* and *E. globulus* may generate information about the genetic basis of the regulation of substitution of xylans.

7.1.2 Pulp

The presence of hemicelluloses is required in pulp used for paper manufacture but they are not desired in the dissolving pulps used to produce acetate products. Both xylans and mannans improve the qualities of paper but mannans are believed to have a greater impact than xylans on the tensile strength of the paper (Wise and Lauer, 1962).

The main polysaccharides present in the card produced from *Eucalyptus* spp. pulp were cellulose and glucuronoxylan. There may also have been some mannans present but this was not determined absolutely owing to the incomplete resolution of xylose and mannose by HPLC.

Any alteration in the biosynthesis or structure of the polysaccharides present in the pulp may affect the properties of the final product. It may be possible to engineer a species of tree for a specific end product. A eucalypt with decreased xylan or with xylan that was easier to extract could be used for dissolving pulps; a line with altered xylan structure, such that it bound

less strongly to lignin, would possibly have lignin that was easier to extract from the cellulose pulp.

7.2 Screening of *A. thaliana* lines

7.2.1 T-DNA tagged lines

Previously mutagenised *A. thaliana* lines from the NASC (all T4 generation) were screened for an altered ability to accumulate cell wall polysaccharides. T-DNA tagged *A. thaliana* lines were screened so that when a line with an interesting mutation in one or more of the screen products was found, the T-DNA tag would be of use in isolating the gene involved. The T-DNA tagged lines selected for the screening process had already been identified as general "form mutants" by either the person who produced the lines or the NASC. The use of form mutants served as a preselection and may have helped to reduce the number of lines that needed to be screened in order to find one cell wall mutant.

7.2.2 Growth conditions

A long day length was used for the growth of the *Arabidopsis* plants so that the mass of tissue produced as the bolting stem would be as great as possible. Also, the stems were cut once the first siliques had formed, again so that the maximum amount of growth had occurred before harvesting and the bolting stems were all harvested at the same development stage. The stems were weighed as soon as possible and then frozen.

7.2.3 Alcohol-insoluble residue

The AIR was chosen as the substrate for the screens as the preparation was quick and a large number of samples could be processed at once. The other components of the AIR, apart from starch, could be ignored. Flowering stems were used because of the presence of secondary xylem.

7.2.4 Screening methods

The screening methods used were Driselase digestion and TFA hydrolysis. Driselase is a crude fungal enzyme preparation and contains a variety of endo- and exo-hydrolases (Fry, 1988). Driselase digestion of the AIR produced monosaccharides and disaccharides characteristic of the major cell wall polysaccharides as well as some unidentified products. TFA hydrolysed all non-cellulosic cell wall polysaccharides, producing monosaccharides Comparison of the products of both screens allowed conclusions to be drawn as to the source polysaccharide of each product.

7.2.5 Initial screen

As an initial screen the products of the assays were separated using PC (Driselase screen) and TLC (TFA hydrolysis screen). The mutants were scored relative to *A. thaliana* ecotype Columbia for each of the screens.

Both PC and TLC are low technology and easy to carry out. A large number of samples can be analysed at once and good resolution of all the major screen products was achieved. PC and TLC are also more tolerant of impurities than HPLC so samples do not have to be as rigorously prepared as for HPLC.

A large proportion of the lines screened (41% for TFA and 43% for Driselase) were identified as showing an appreciable difference from Columbia in the quantities of one or more screen products. The large percentage identified could be partly due the preselection of lines previously categorised as form mutants. Also, the line referred to as the wild type for the initial (and repeat) screen was Columbia. Columbia is the parental line for the Koncz lines; while Landsberg *erecta* is the parental for the Bancroft and Dean lines and Wassilenjka (Ws) is the parental for the Feldmann lines. The HPLC analysis of the screen products of Ws and Columbia showed that there were some differences between the two lines in the yield of cellulose- and starch-derived glucose with only minor differences in the other screen

products. Landsberg *erecta* was not analysed by HPLC owing to difficulties with germination.

The majority of the observed differences in the screen products were in xylose (TFA screen) and xylobiose (Driselase screen). Most of the observed differences were decreases compared to Columbia. This could be due to the nature of the lines screened. They were all generated by the insertion of a piece of foreign DNA (T-DNA tag or a transposon) into the plant's genome. This would disrupt the function of the gene and be observed as a decrease in the level of one or more screen products.

7.2.6 Repeat-screen

Owing to time constraints not all the lines identified by the initial screen were repeat screened. Ten of the 44 lines identified by the TFA screen and 18 of the 41 lines identified by the Driselase screen were repeat screened. Of the lines that showed consistent differences from Columbia a small number (three out of five for the TFA screen, and five out of nine for the Driselase screen) showed differences that were at least partially consistent with those observed in the initial screen.

7.2.7 HPLC

Nine lines (all Feldmann lines), identified by the repeat screen as showing a consistent difference from Columbia in one or more of the screen products, were analysed by HPLC. All the lines showed differences from Ws in either the glucose- or the xylose-containing polysaccharides.

As the nine Feldmann lines were all genetically heterogeneous (Section 2.2.1.2) statistical comparisons were made between individual plants and the Ws group (Section 2.2.6).

For Ws, 66% of the xylose in the AIR was Driselase-resistant. This was probably due to substitution of the xylose residues with acetyl groups or (methyl)glucuronic acid. Four of the lines (B5, B24, B27 and B58) had at least

80% of individuals with significantly different levels of Driselase-susceptible xylan than Ws. The levels of xylobiose in the Driselase products of these lines were 72-132% more than Ws for B5, 25-65% less than Ws for B24, 58-68% more for B27, and 39-46% more for B58. These results suggest that the level of substitution of the xylose residues in xylan (with [methyl]glucuronic acid residues, arabinose residues and acetyl groups) was lower than in Ws.

The total level of xylan (TFA-susceptible xylose) was lower (by 27-37% of the Ws level) in 71% of B52 individuals and the proportion of Driselase-susceptible xylobiose was significantly higher (by 39-51%) than in Ws in 57% of B52 individuals. This suggests that there was less xylan present in the majority of the AIRs of B52 than in Ws and the level of substitution of the xylose residues (with [methyl]glucuronic acid residues, arabinose residues and acetyl groups) was higher than Ws.

At least 86% of individuals from each of the nine Feldmann lines had reduced levels of unidentified peaks, which could also be due to substitution of xylose residues (with [methyl]glucuronic acid).

Not all the products of TFA hydrolysis and Driselase digestion were identified. This does not invalidate the results. The comparisons between the identified products still indicate the susceptibility of the polysaccharides in the AIR to the two screening methods and hence have implications for the structure of the polysaccharides. The unidentified products are probably di- or trisaccharides, formed by partial breakdown. In the TFA hydrolysates there may be oligosaccharides of galacturonic acid and rhamnose, and glucuronic acid and xylose, because uronic acid glycosyl residues are relatively resistant to acid hydrolysis.

The unidentified peaks on HPLC of the Driselase digestion products have yet to be identified with certainty. This was not accomplished owing to a lack of suitable markers for the proposed compounds. An alternative strategy would be to extract xylan from wood (or use commercially available birch wood xylan) and digest it with Driselase, separate the products by preparative HPLC, PC or TLC, hydrolyse the compounds of interest and

then run the products on HPLC, PC or TLC to identify the monosaccharide components.

7.3 *Arabidopsis* genetics

It was not conclusively shown whether the T-DNA tag(s) of the identified lines co-segregate with the observed differences in the screen products. The next step would be to repeat this section to see if the T-DNA tag and the observed difference in screen products co-segregate. The tag could then be used to locate the region of the genome of the mutation resulting in the observed difference in screen products.

7.4 Conclusions

Xylan forms a significant proportion of the mass of processed pulp and can have a major impact on its properties. Xylan is only a minor component in the primary cell walls of dicots (approximately 5%; Reiter, 1994) but in secondary walls it is present at much greater levels (20-30%; James *et al.*, 1985). It is possible that small changes in the level and/ or composition of the xylan found in the AIR of the *A. thaliana* lines may, once transferred to *Eucalyptus,* where xylan makes up a larger proportion of the total cell walls, affect the properties of the pulp produced from the affected trees. The proportion of xylan in the AIR will be lower in *Arabidopsis* than in *Eucalyptus* owing to the presence of non-xylem cell types, while in pulp production, the majority of the tissue used will be secondary xylem. Cork and phloem layers will be removed prior to pulp production, and cortex, pith and epidermis will be negligible in *Eucalyptus*

Sometimes it would be desirable to leave some hemicellulose in the pulp but the hemicellulose also affects the retention of residual lignin (use of a xylanase preparation can improve the brightness of the paper while not directly affecting the lignin content). A change in the structure of xylan (loss of moieties that form linkages to lignin) could decrease the amount of

residual lignin retained by the pulp, and hence decrease the number (and severity) of bleaching stages required to extract most of the lignin. Excess bleaching stages (and the associated alkali washes) adversely affect the tensile strength of the pulp by degrading the fibres.

It is hoped that these lines will help to illuminate the (regulation of the) biosynthesis of cell wall polysaccharides. Analysis of the composition of the mutants' cell walls may help show how the biosynthesis of the polysaccharides is regulated and how a defect in the biosynthesis of one polysaccharide may affect the biosynthesis of another. For example, Shedletzky *et al.* (1990, 1992) found that in tomato cells grown in the presence of the herbicide, 2,6,-dichlorobenzonitrile, so that the cell wall virtually lacks a cellulose-xyloglucan network, the pectins were capable of forming a wall structure sufficient for cell growth and structural integrity.

A small number of T-DNA tagged *A. thaliana* lines have been identified that show significant differences from the parental line in the composition of the screen products. These lines show differences in the level of susceptible xylan, substituted xylose residues, cellulose and xyloglucan. These differences are relatively small and, if transferred to *Eucalyptus*, may affect the qualities of the pulp. However, as each of the lines was generated by the insertion of a T-DNA tag into the genome, there is a possibility that the T-DNA insertion event caused the observed mutation in a gene for cell wall composition. If this is so the T-DNA tag can be used to locate the gene in question. Any information about the genes involved in the synthesis and activation of monosaccharides, transport in to the Golgi vesicles, synthesis of cell wall polysaccharides, incorporation of these polysaccharides into the cell wall is of use in the struggle to examine qualitatively and quantitatively the biosynthesis of the plant cell wall. While these initial defects may not affect the quality of pulp, they may be of use in the search for the genes involved in the biosynthesis of the plant cell wall and may lead to further understanding of the biosynthesis of the plant cell wall. Such information

could then lead to genetic modification of *Eucalyptus* trees in such a way as to affect the properties of the pulp produced.

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